PRODUCTION, PURIFICATION AND CHARACTERISATION OF L-ASPARAGINASE FROM ACTINOMYCETES AND EVALUATION OF ITS ANTI-CANCER AND ACRYLAMIDE REDUCTION ACTIVITIES

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CERTIFICATION

I certify that this work was carried out under my supervision by M.O Salami in the Department of Microbiology, University of Ibadan, Nigeria

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DEDICATION

I dedicate this work to Almighty God, my pillar, enablement, and only source of strength, the Alpha and Omega.

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ABSTRACT

The potential of L-asparaginase to inhibit acrylamide formation (a carcinogenic compound from fried starchy foods) and the growth of cancer cells have attracted scientific attention in biomedical fields. Utilisation of L-asparaginase from several sources has been limited due to high rate of allergic reactions, after a prolonged use because of the presence of glutaminase activity which deamidates glutamine to glutamic acid and ammonia, thereby increasing the enzyme toxicity. Hence, it is necessary to search for other L-asparaginase sources with no L-glutaminase activity as alternatives. Production trials with Actinomycetes and their characterisation have been limited. The work aim was to produce, purify and characterize L-asparaginase with anticancer and acrylamide reduction potential from Actinomycetes.

Actinomycetes were isolated from rhizospheric soil of *Moringa* plants in the botanical garden, University of Ibadan, and were screened for L-asparaginase activity. The selected L-asparaginase producers were screened for L-glutaminase activity using plate assay method. The L-asparaginase producers with lower glutaminase activity were identified using molecular methods. Effects of temperature, pH, metal ions, incubation time, carbon and nitrogen sources, agitation and medium composition on the growth and L-asparaginase production by identified isolates were determined. The L-asparaginase produced was purified by ammonium sulphate precipitation, dialysis and column chromatography using Sephadex G-50. The molecular weight of the enzyme produced was determined using SDS-PAGE. Effect of pH, temperature, metal ions, inducers and inhibitors and substrate concentration on activity and stability of the partially purified enzyme was determined. The *in-vitro* anticancer activity of the partially purified L-asparaginase on colon cancer cell line at different concentrations using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was done. The acrylamide reduction activity of L-asparaginaseinpotato after heat treatment (above 120^{o}C) was determined using standard methods. Data were analysed using descriptive statistics.

One hundred and forty-five bacteria were isolated, 46% of them were L-asparaginase producers. The best six L-asparaginase producers with lower glutaminase activity were identified as *Amycolatopsis japonica*, *Sphingobium yanoikuyae*, *Sphingobacterium caenis*, *Stenotrophomonas pavani, Paenibacillus cineris and Actinomycetal bacterium*. *Amycolatopsis japonica* showed highest L-asparaginase activity (0.2772±0.001 U/mL) and no glutaminase activity after 25 days of screening. The highest L-asparaginase was produced at pH 7.0, 35^{o}C, 7 days incubation time, Mg^{2+}, 150rpm, M9 medium, maltose (0.2% w/v) and yeast extract (0.2% w/v). Partially purified L-asparaginase from *Amycolatopsis japonica* had total activity of 1968.98 U/mL, total protein (26.69 mg), specific activity (73.75 U/mg), purification fold (6.42), recovery yield (42.86%) and molecular weight of 37.5 kDa. Ethylenediamine Triacetic Acid, Mg^{2+}, pH 8, 45^{o}C supported optimum activity and stability of the enzyme. The Michaelis constant (K_m) and maximum velocity (V_{max}) were 7.874 mM and 2.57 U/mL, respectively. The enzyme showed high anticancer activity against colon cancer cell line with half Maximum Inhibitory Concentration (IC_{50}) of 36 µg/mL. It also had 11% reduction of acrylamide formation in potato which is a carcinogenic compound in starchy food.

Amycolatopsis japonica L-asparaginase exhibited anticancer potential and reduced formation of acrylamide in fried starchy food. This enzyme has the potential to be used as drug to complement the ones currently in use.

Keywords *Amycolatopsis japonica*, Glutaminase activity, Anticancer activity, L-asparaginase

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CHAPTER ONE
INTRODUCTION

1.1. Background to the study

Microbial metabolism yields a lot of metabolites which could be active within the millieu of production or utilisable externally as a value added product. Different bioactive compounds have been found to be produced by microorganisms, such as antibiotics, bio-pesticides and enzymes (Nikel, 2016). Enzymes are refered to as proteins or biomolecules which speed up chemical reactions. In the reactions, 'substrates' (the molecules at the beginning of the reaction) are changed into diverse molecules, which are referred to as 'products'. Enzymes are more important than vitamins and minerals for general health (Darwesh *et al.,* 2020) and occur naturally, being produced by living organisms and functioning as biochemical catalysts. Recently, in the medical field, different enzymes have been given much attention as they are being used as drugs; L-asparaginase is one of these enzymes and is widely present in nature.

Actinomycetes are Gram positive bacteria that have high G + C content in their DNA, a thread- like colony presentation that look like fungi, in that they form branching filament, reproduce asexually, have the ability to produce spore and mycelia structures (Dhanam and Kannan, 2015). Several useful metabolites have been reported to be produced from this group of bacteria, which makes them to attract the attention of scientists and also find use in agricultural industries, pharmaceutical industries and in medical fields (Kumar *et al.,* 2017). Several reports have been recorded on production of antibiotics from actinomycetes, but

little studies have focused on enzymes production, especially L-asparaginase, from this group of bacteria (Bhargavi and Jayamadhuri,2016).

Development of diseases in human will continue with the speed relative to experimental and clinical science improvements (Pan *et al.,* 2018). This makes it more imperative for biological researchers worldwide to search for newer drugs that can be used to prevent and combat some terminal diseases, like cirrhosis, renal dysfunctions and cancer diseases. Actinomycetes such as, *Streptomyces longsporusflavus, Streptomyces karnatakensis,* marine *Streptomyces* sp PDK2, *Streptomyces griseus, Streptomyces venezuelae, Streptomyces gulbargensis,* and *Nocardia* species (Dhanam and Kannan, 2015) have been reported to be L-asparaginase producers.

Among the largest group of enzymes used as therapy in the medical field, L-asparaginase accounts for up to 40% of the sale globally, and it has gained much relevance because of its use in the treatment of leukemia, lymphocytic leukemia and different kinds of cancer in man (Sanjotha, 2016, Alruman *et al.,* 2019). L-asparaginase is a hydrolytic enzyme, it functions by physiologically irreversibly breaking down L-asparagine (an amino acid), to L-aspartic acid and ammonia (Sanjotha, 2016). This enzyme is made of four subunits which are identical and catalyses the reaction by gripping it active sites on L-asparagine.

L-asparagine is a common natural amino acid, and is classified as non essential amino acid since it can be produced by the body cells (Dhanam and Kannan, 2015). L-asparagine is so important for tumor cells to get their protein, so as to survive, whereas the normal cell can grow without its requirement, as normal cell has an enzyme called L-asparagine synthase, which help them to produce the protein within the cells and can be absorbed from the outside. This makes the cancer cells more different from normal cells as they are incapable to synthesize L-asparagine because they do not have L-asparagine synthetase, thereby depending on the ones in the circulating plasma pools (Dhanam and Kannan, 2015). Mean while, when L-asparaginase is present, cancer cells cannot access an essential growth factor (L-asparagine) which is essentially needed for their growth, thereby limiting their growth, since they are unable to produce L-asparagine in their cells to foster their continuous growth. Supplementation of this enzyme leads to continuous reduction of L-asparagine.

For L-asparaginase to be therapeutically useful, its isolation must be easy, must be stable at physiological temperature and pH, its K_m value must be low and must not

be inhibited by its substrate, substrate affinity of the enzyme is responsible for its effectiveness on cancer cells (Lincoln *et al*., 2021).

L-asparaginase is used in the medical field for the treatment of leukemia, divese kinds of cancer and is also important in food and baking industries, to reduce the formation of acrylamide (a carcinogenic compound) in fried carbohydrate food (Ushakiranmayi *et al.,* 2017). It is accessible in the market under brand names like Aspartase, Preventase and Erwiniase. Also, it is extensively involved in the ultilisation and metabolism of amino acid in the body. It helps in transamination reaction that form oxalo acetate which leads to glucose formation via glucogenic pathway and also serve as precursor othinine in the urea cycle (Cunha *et al*., 2018).

L-asparaginase has been found in microorganisms, plants and animals. Filamentous fungi, yeast, actinomycetes and bacteria has been a microbial sources of this enzyme, which has gained so much relevance since it is extracellular and can be produced from simple fermentation medium, the medium for it production can be optimized easily, and can be genetically modified easily to give high yield, the extraction and purification of the enzyme can be done with little cost, it can be produced in large quantity, and tend to have good stability and consistency than those from other sources (Lincoln *et al*., 2021).

1.2. Statement of problem

L-asparaginase has been reported to be effective in limiting L-asparagine availability in the serum and therefore being therapeutically active in the management of lymphoid tissue-related disorders such as lymphoblastic leukemia and malignant disorders. Despite its effectiveness, production limitations have always been encountered, especially with the presence of contaminating traces of L-glutaminase. The L-glutaminase enzyme reduces the L-asparaginase purity and affinity and can lead to high rates of allergic reactions such as blood clotting, anaphylaxis shock, and rashes after prolonged use due to increase in the enzyme toxicity.

1.3 Justification for the study

It is therefore necessary to search for high L-asparaginase-producing microorganisms with very minimal (or completely devoid of) L-glutaminase content so as to prevent these adverse immunogenic effects. Actinomycetes (with

no glutaminase activity) may be a better source of L-asparaginase and these were thus investigated.

1.4. Aim and Objectives

1.4.1. Aim

This work aims to produce, purify and characterise L-asparaginase with anticancer and acrylamide reduction potentials from Actinomycetes.

1.4.2. Specific objectives:

a) Isolation, screening and identification of L-asparaginase-producing strains from plant rhizospheres.
b) To study the effect and optimization of production parameters of L-asparaginase from selected strains.
c) Production, purification and characterisation of L-asparaginase produced by the selected strains.
d) Application of L-asparaginase produced, on cancer cell lines and acrylamide reduction in food sample

CHAPTER TWO
LITERATURE REVIEW

The importance of microorganisms cannot be overemphasized as they are widely spread in nature. They are the largest of all the organisms on earth and exist almost everywhere. They contribute maximally in occurrence of nature and their functions in provisions of life essential elements cannot be underestimated (Yang *et al*., 2020). At the beginning of all ecological food chains, microorganisms are said to be the source. Some of them are harmful while some are beneficial. Those microorganisms that inhabit the human body also contribute in the digestive system of the body and in producing some vitamins. Also they are important in pharmaceutical, food and brewery industries (Yang *et al*., 2020).

Microorganisms are diverse, some can be motile and some non-motile, some microorganisms have cell walls and are photosynthetic like plants. Some have true nucleus (eukaryotic cells) while some have false nucleus (prokaryotic cells). An example of a prokaryotic cell microorganism is bacteria, which are single-celled and have peptidoglycan in their cell walls. They exist abundantly in the air, water, animal dung and mostly in soil. Some are normal flora of the mouth, skin and intestine while some live in extreme environment such as high temperature, high sanitation, and high pH. Examples of eukaryotic cells are fungi which are multicellular and contain hyphae (a thin, threadlike structure) through which they absorb the nutrients that they utilise as their source of energy and carbon from the environment (Kumar *et al.,* 2013).

2.1 Actinomycetes

Microorganisms from the soil provide good resources for the isolation and identification of some important therapeutic products, one of such microorganisms are Actinomycetes. Actinomycetes are unicellular organisms that forms branching filament called hyphae. They reproduce

asexually either by special spores or by fission (Yang *et al*., 2020). They resemble bacteria and are called higher or filamentous bacteria. They are Gram-positive bacteria, with their DNA consisting of high guanine and cytosine. They belong to Order A*ctinomycetales* (Sivasankar *et al.,* 2018). They are unique among other bacteria, because they have the potential to produce branched network of mycelia and have large range of life cycles (Subramani and Sipkema, 2019).

Several metabolites that are important in the agricultural and medicinal field have been known to be produced by filamentous bacteria (Silva *et al.,* 2017). They have some distinct characteristics which make them good producers of metabolites that are biologically active, such as antioxidant reagents, immunological regulators, enzyme inhibitors, enzymes and antibiotics and which are therapeutically useful compounds. Actinomycetes can be found everywhere in nature, especially soil and ocean (Yang *et al*., 2020). They decompose organic matter in soil, such as chitin, starch and lignocelluloses. They help in the rhizosphere as they protect plant roots against infection by pathogens and also aid plant growth. They are used as agents for biological control of root-borne disease of crops and plants (Dimri *et al*., 2020).

Their isolation is tedious due to the fact that they grow slowly. The discovery of massive unculturable microbial diversity) did not change this view as prolific producers of bioactive compound were readily isolated but not identified as significant in molecular ecologic studies (Dimri *et al*., 2020). Nevertheless, better identification, confirmed isolation and successful screening of new novel bioactive compounds all indicate that novel biology and chemical diversity is holden in the complex microbial communities, heterogeneously distributed in time and space (Dimri *et al*., 2020).

Actinomycetes are divided into phylum; Actinobacteria, class; Actinobacteria, subclass Actinobacteridae, and order Actinomycetales. It has 10 and 30 suborders and families respectively and consists of 160 genera (Subramani and Sipkema, 2019). Out of these general, it has been shown that only a few account for large bioactive compound discovered such as immunosuppressant and antitumor agents and antibiotics (Alvarado *et al.,* 2018). Also, of the entire bioactive products from Actinomycetes, larger percentage of it was from the genus *Streptomyces* (Kamjam *et al.,* 2017).

It has been reported that actinomycetes are so much important which make them to be one of the pioneer microorganisms that had their genomes sequenced (Vongsangnak *et al.,* 2012). When genome of different actinomycetes were sequenced, it was reported that, actinomycetes contain huge number of unknown biologically active compounds and the genes are larger than other bacteria (Lyddiard *et al*., 2016) for example *Streptomyces ambofaciens* was genome-mined because it has a number of cryptic (unstudied) gene clusters and produced congocidine and spiramycin (Gutierrez *et al.,* 2017).

Table 2.1: Classification of actinomycetes

Group	Characteristics
Nocardioform Actinomycetes	They are aerobic organisms, formed branched filaments, acid-alcohol fast; are rods, cocci shape, or form mycelium that fragment, contain mycolic acids.
Actinomyceteswith multilocular sporangia	Their mycelium splits in all planes, they do not have aerial hyphae, wall chemotype and are aerobic to facultatively anaerobic.
Actinoplanetes	They cannot move, have no aerial mycelium, they are aerobic organisms, their spores may be convered inside vesicles, have xylose and arabinose in it hydrolysates and produce spore, with wall chemotype ii;
Streptomycetes	They produce spores and form both aerial and substrate mycelium and grow aerobically.
Thermomonospora and related genera	They grow aerobically and can produce spore which can either be motile or non-motile.
Thermoactinomycetes	Thermophilic and contain meso-Dap in their DNA, but has no sugars or amino acids. They produce single spore on their vegetative and aerial filament.
Some other genera	They produce chains of spores with aerial growth.

Source: (Dimri *et al*., 2020).

2.1.1 Distribution of Actinomycetes

Various habitats harbour actinomycetes, but mostly, they are found in the surface layer of the soil (Kumar and Jadeja, 2016). The types and percentage of Actinomycetes in soil depends on environmental factors. Jacob and Sudini, (2016) reported that, out of twenty genera of actinomycetes isolated from soil, 95% are streptomycetes. Most Actinomycetes grow in a medium with neutral pH. They grow well between pH range of 5.0 and 9.0, and maximally at pH 7.0. pH is very important, as it determines the occurrence and bioactivity of Actinomycetes in soil. Most Actinomycetes cannot grow well in soils with pH below 5.0, but there are some exceptions, acidophiles like acidoduric and acidophilic streptomycetes is much in soils with low pH. Most Actinomycetes in the Laboratory express themselves as mesophiles and grow well at temperature range between 25 - 30^{o}C (Dimri *et al*., 2020). They help in decomposition of organic matter. However, the ability to decompose organic matter makes some to be able to withstand high temperature above 40^{o}C; these are called facultative or obligate thermophilic actinomycetes e.g. thermo-actinomycetes and saccharomonospora. Thermophilic actinomycetes are able to grow well on manure from animal and are involve in deodorization of pig faeces and in straw fermentation.

Actinomycetes can decompose plant and degrade some complex polymers such as lignocelluloses, lignin and cellulose. They also degrade polymers that occur naturally in soil, for example; chitin, keratin, pectin, fungal cellwall material and hemicellulose. They are found in the rhizospheric soil and are able to synthesise gibberellins-like substances, hydrolyse starch and inhibit the growth of plant root, for example, Streptomyces that inhabit the rhizosphere controls fungal root pathogens (Jacob and Sudini, 2016).

Many inhibitory interactions apart from antibiotics may happen between fungi and Streptomyces. Streptomyces in the rhizosphere have been reported to be responsible for the reduction in the incidence of infections from plant pathogen due to the production of antifungal antibiotics (Dimri *et al*., 2020).

The discovery of new secondary metabolites from terrestrial habitat is reducing, However, there is increase in the rate at which new compounds are isolated (Subramani and Sipkema, 2019). It is very important to isolate new strains of

actinomycetes so as to discover new compounds that have pharmaceutical properties (Kamjam *et al.,* 2017). The occurrence of predominant group and less occurring microorganism depends on different types of environmental factors. These factors help to change the inhabitant microorganism chemical makeup (Chaurasia *et al.,* 2018).

From the studies of isolation of actinomycetes from soil, it has been reported that the most abundant genus isolated are *Streptomyces* and *Micromonospora* (Yellamanda *et al.,* 2016). Farming soil-actinomycetes which were antagonistic against few bacteria and had capability of being a producer of novel antibiotics was discovered by Oskay *et al*., 2004 as reported by Myla (2016). Recently, Hong *et al.* (2009) isolated *Streptomyces* and *Micromonospora* that have the ability to produce bioactive compound. Tidal influence makes the actinomycetes in the mangrove rhizosphere to be more than that in the arable lands. They are majorly, *Streptomyces, Micromonospora,* Actinobacteria and Nocardioform actinomycetes (Kamjam *et al*., 2017). A study conducted by (Schneider *et al.* (2007) reported nocardioform actinomycetes from mangrove soil was isolated, which have capacity to produce cytototoxic metabolites that inhibited adenocarcinoma cell line (Shaaban *et al.,* 2017).

Actinomycetes inhabit plant surfaces and various parts of plants, especially on the root closed to the soil. Several strains of actinomycetes were isolated by Tian *et al.* (2004) on rice's sterilized root and leaves. Most of them belonged to *Streptomyces sp,* only a few fell into *Streptoverticillium* (Kampapohgsa and Kaewkla, 2016). In another study, Petrolini *et al.* (1995) reported that 1755 actinomycetes strains was isolated from surface-sterilized roots of 205 plants from 156 species. They identified eighty percent of the strains as *Streptomyces* spp. and the remaining as Nocardioforms, *Micromonospora, Actinoplanes, Streptosporangium, Streptoverticillium* and *Saccharomonospora* (Alvarez-Lopez *et al.,* 2020). Coombs and Franco (2003) isolated endophytic actinomycetes from healthy wheat plants roots. They found that the isolates belonged to *Streptomyces, Microbispora, Micromonospora* and *Nocardiodes* (Chaurasia *et al.,* 2018). Taechowisan *et al.* (2003) isolated endophytic actinomycetes, particularly *Streptomyces* sp with antifungal potential healthy tissues leaves, stems and roots (Jha and Modi, 2018)

2.1.2 Aquatic habitat and Actinomycetes

2.1.2.1 Freshwater habitat

Actinomycetes are abundant in fresh water lakes (Naaz *et al.*, 2020). It was reported that thermophilic actinomycetes are found in river water (Alzahrani *et al.*, 2020). They were also found in sewage and grew well at 60˚C. Cross, (1982), proved this statement by isolating members of genera *Rhodococcus, Streptomyces, Micromonospora,* endospore-forming *Thermoactinomycetes* and *Actinoplanes* from freshwater habitats. Majority of these actinomycetes most probably are wash-in from land and accumulated in freshwater habitats (Alzahrani *et al.*, 2020). According to Makkar and Cross (1982), sporangia of *Actinoplanes* could withstand prolonged dessication and release motile spores when rehydrated. That is why they are common in soils, rivers and lakes. *Actinoplanes* is mainly discovered on allochthonous leaf litter washed to lake shore and twigs submerged in streams (Kurtboke, 2017).

Waksman (1959) believes that members of the genus *Micromonospora* represent a truly indigenous group of microbial inhabitants of waters and bottom deposites of inland lakes. Therefore, a great number of *Micromonospora* also can be isolated from lake sediments. Complex organic compounds such as chitin, cellulose and lignin accumulated in lake mud were reported to be decomposing by this genus of Actinomycetes (Ahmad *et al.,* 2016). Rowbotham and Cross (1977) elucidated that *Micromonospora* spores can survive as dormant propagules as they washed into streams, rivers and lakes (Souza *et al*., 2020).

Also, the existence of *Rhodococcus coprophilus* (which has been isolated from water and sediments of rivers and lakes), a coprophilic species in lakes was believed to be due to wash in of contaminated herbivore dung (Sousa *et al*., 2020). A study of freshwater actinomycetes isolation from sediments of lakes was carried by Jiang and Xu (1996) resulted in the discovery of predominantly *Micromonospora* followed by *Streptomyces*. The presence of *Streptomyces* in freshwater habitat was because of their spores being continuously washed into rivers and lakes. This enables the *Streptomyces* spores to be found in foams of rapids at the water-air interface (Ma *et al*., 2020). Isolation of actinomycetes from lake water and sediments by Terkina *et al.* (2002) leads to an interesting conclusion that

Streptomyces were dominant in water samples while great numbers of *Micromonospora* were found in sediments (protasov *et al.*, 2017).

2.1.2.2 Marine habitat

Marine habitat has some characteristic features that makes them different from the terrestrial habitats, like high salt concentration, low concentration of organic matter and high hydrostatic pressure. Therefore, the microorganisms that will strive thus, microorganisms surviving both habitats will be different. This makes a point of view that marine habitat would be an excellent sampling point as microbes in seawater and marine sediments are diverse. Millions of species of microorganisms, actinomycetes inclusive, lives in the marine habitat. Microorganisms help in the decomposition of plant, animal and dead plankton, they mineralize complex organic matter and help in degradation of toxicants and pollutants. They are good source of primary and secondary metabolites (Chaurasia *et al.,* 2018). From early study, it was reported that the presence of actinomycetes in marine habitat was due to soil contamination, presence of algal material floating on the surface of the sea, or due to collection of sample near the dock (Dimri *et al*., 2020).

New and different types of actinomycetes are present in marine environment. The first marine actinomycete was isolated in 1991 and was named 'Salinispora' (Maldonado *et al.,* 2020). It was reported that strains of *Salinospora* are been found around the globe which includes Pacific Ocean, carribean and Gulf of Mexico. Jensen *et al.,* (2007) reported three species of *Salinispora*, they are *S. pacifica*, *S. tropica and S. arenicola*, and reported that there pattern of production of secondary metabolites is species specific (Millan-Aguinaga *et al.,* 2020). One of the bioactive compounds produced from the genus *Salinispora* is Salinosporamide A**.** and the compound is currently in clinical trial stage to be use against tumor cells (Dhakal *et al.,* 2017).

Several researchers have isolated new useful bioactive metabolites from actinomycetes from marine habitat, this suggests that these microorganisms serves as new source of natural products from marine habitat (Sousa *et al.,* 2020). Some studies reported that there was no difference in morphology and biochemical features between marine and terrestrial actinomycetes, and suggested that, they were initially from terrrestial environment but later adapted to salinity level of sea water (Dimri *et al.,* 2020). They studied the transportation of actinomycetes spores

into shallow sea mud. The study explained that actinomycetes spores might have been washed off from land to the ocean by river or rain and survived (Dimri *et al.,* 2020). Spores were precipitated by the sodium chloride acceleration after reaching the sea into sediments.

Most of the actinomycetes gotten from marine habitat have adapted to the sea, and have the capacity to produce active metabolites. It was reported based on further studies on isolation, that actinomycetes from marine sediments has the ability to withstand high salinity, which makes them to be able to withstand marine conditions and survive (Kamjam *et al.,* 2017). Marine actinomycetes isolated from sediments counts far exceeded those found in sea water. Thus, sediments are still the best supplier of this microorganism (Dimri *et al.,* 2020). A total of 192 marine isolates were isolated from sediments, they were identified and resulted in the discovery of novel marine-derived actinomycetes within the family *Micromonosporaceae* (Kampapohgsa and Kaewkla, 2016). All these studies proved that marine sediments are really a valuable source for isolating marine actinomycetes (Sharma *et al.,* 2019).

2.1.2.3 Extreme environments

Studies have shown that some actinomycetes can be isolated from extreme or uncommon habitats. A new genus described as *Bogoriella caseilytica* has also been identified on alkaline soil (Dhakal *et al.,* 2017). Those actinomycetes that are able to withstand alkaline pH are refers to as Alkalophilic actinomycetes, for example, *Streptomyces* and *Nocardiopsis* had been isolated from alkaline soils (pH 10-12) surrounding mineral springs. Also, Halophiles (organisms that are able to withstand high salinity) "*Saccharomonospora halophila"* from soil containing up to 10% sodium chloride was reported to be have been isolated (Bhatti *et al.,* 2017). Psychrophile (organisms that grow at lower temperature) like *Modestobacter multiseptatus, Cryobacterium psychrophilum with* optimum growth temperature 9-12˚C were isolated from transantarctic mountain and Antartic soils respectively (Ahmad *et al*., 2017). Besides, Zakalyukina *et al.* (2002) isolated acidophilic actinomycetes, (that is, organisms growing at lower pH range) from acidic soils, mainly *Streptomyces* and *Micromonospora*. *Microbispora, Nocardia, Microtetraspora, Amycolatopsis, Actinomadura* and *Saccharothrix* were reported

to be isolated from desert soils of Mojave Desert as they were able to withstand high temperature of about 50^{o}C (Zakalyukina *et al*., 2017).

2.1.3. Mode of feeding and features of actinomycetes

Actinomycetes are heterotrophs, they can be parasitic, saprophytic and some form mutualistic association with plant and animals. They play major role in recycling of nutrient in the ecosystem. They mostly grow aerobically, though there are exceptions like actinomyces that grow anaerobically. Most actinomycetes grow on general medium used in the laboratory for their growth, but some like *Frankia* species and sporoactinomycetes need special media and incubation condition so as to allow growth differentiation and produce unique features like production of pigment and spore formation (Zeng *et al.*, 2019).

Most of the media used for cultivation of actinomycetes are not commercially available; hence, they are prepared using different types of organic and inorganic material in the laboratory. The appearance and formation of actinomycetes colony on the cultivating medium is media dependent. For example, *Streptomyces species* give hard, tiny, pale, shiny colonies on nutrient agar and appeared differently when grown or subculture on another growth medium that is more suitable, for example, starch agar or oatmeal. They develop an outgrowth (fragments of mycelium) which project and develop into a hyphae. The hyphae form substrate mycelium that penetrates into the medium to form leathery or tough colony. The medium composition determines the consistency and density of the colony (Zeng *et al.*, 2019).

2.1.4. Roles attributed to actinomycetes in ecosystem

Significant numbers of Actinomycetes are present in the ecosystem and form major component in most soils. They are mostly saprophytic in nature and functions expressly in decomposition of organic matter (Dimri *et al.,* 2020). Goodfellow and Williams (1983) reported that, Actinomycetes play a crucial role in degradation of plant litter, mostly in the recalcitrant lignocelluloses component. Since they form a resting or dormant spore, they germinate in the presence of exogenous nutrient; there are also some environmental factors that affect actinomycetes growth, such as, pH, soil temperature and moisture content of the soil (Dimri *et al.,* 2020).

Actinomycetes found in the rhizospheric soil have the ability to produce antibiotics and other metabolites, which in turn repel the pathogens in the rhizosphere. The ability of *Streptomyces* spp. to biologically control some pathogenic fungi affecting commercial plants (Chavan *et al*., 2020). It was reported that *Streptomyces* spp. isolated from grapes have antifungal potential against pathogenic yeast and fungi from grape. Though, there are some pathogenic Streptomyces which causes plant disease. For example *S. turgidiscabies, S. luridiscabiei, S. puniciscabiei* and *S. niveiscabiei which causes* scab lesion on potato (Yadav *et al.,* 2017).

2.1.5. Identification and characterisation of actinomycetes

The polyphasic taxonomic approach of actinomycetes is considered to be phenotypic and phylogenetic methods .the phenotypic includes morphological, physiological and biochemical characterisations while the phylogenetic are molecular methods (Tan, 2007).

2.1.5.1 Cultural and morphological observation of actinomycetes

There are different cultivating media that are used in the cultivation of actinomycetes, such as, glycerol – L-asparagine agar (ISP5), oatmeal agar (ISP3), inorganic salts – starch agar (ISP4) and yeast extract – malt extract agar (ISP2),. The morphology of *Streptomyces* spp. is best viewed on a grown and mature culture plate so as to observe the substrate mycelium, diffusible pigment colour and aerial spore mass colour (Bhatti *et al.,* 2017*).*

Based on different spore chain morphology, *Streptomyces* spp. can be divided into seven groups of "morphological sections.

No	Section	Figures	Description

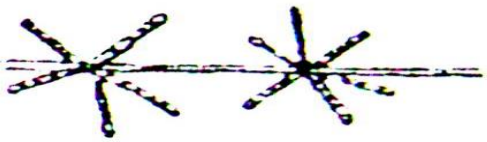
1	Rectus flexibilis (RF)		Straight, flexuous or fascicled spore chains; no spirals
2	Retinaculumapertum (RA)		Hooked, open-looped or greatly extended spiral spore chains
3	Spira (S)		Closed or open spirals
4	Monoverticillus (MV)		Primary verticils or whorls attached to long, straight branches; no spirals
5.	Monoverticillus – Spira (MV-S)		Spiraled primary verticils or whorls attached to long, straight branches
6	Biverticillus (BIV)		Compound verticils or whorls attached to long, straight branches; no spirals
7	Biverticillus – Spira (BIV-S)		Spiraled compound verticils or whorls attached to long, straight branches

Figure 2.1: Seven morphological sections of *Streptomyces* spp. (Bhatti *et al.*, *2*017*)*.

2.1.5.2. Physiological characterisation of actinomycetes

Basically, any actinomycetes physiology consideration entails studying their mode of nutrition and growth, how they can withstand environmental conditions and their

metabolic processes. Carbon sources utilization and melanoid production has been characterized for *Streptomyces* spp. physiology. Moreover, some physiological characteristics to categorize actinomycetes, especially *Streptomyces* spp. had been considered, and this depends on number of isolates and objective of the study. For example, temperature range, nitrate reduction test, NaCl tolerance, production of hydrogen sulfide, starch hydrolysis, liquefaction of gelatin, nitrogen source utilization, pH sensitivity and sensitivity to some antibiotic (Sharma *et al.,* 2019).

2.1.6 New method of isolating Actinomycetes:

The discoveries of unknown and essential natural product can only be achieved by using new method of screening. When comparing the isolation of other bacteria with that of actinomycetes, it has been reported that, isolation of actinomycetes from mixed culture is tedious because, they grow slowly compare to other bacteria. The types of organisms founds in the environment is related to the environmental condition of that area. Differences between actinomycetes isolated from marine and freshwater environment has been reported. Some antinomycetes, are antagonist, for example, xanthomycin-producing actinomycetes, and have been recorded to be isolated more often in the marine habitat than in the soil. Some of these organisms are rare actinomycetes; they produce novel secondary metabolites which are biologically active. Thus, marine habitat seems to be a good source of actinomycetes with bioactive compound (Sharma *et al.,* 2019).

Another means of isolating of new actinomycetes is by employing pre-treatment methods, such as, drying, dehydration or rehydration. Many actinoplanetes, and the new genus 'cupolomyces' has been isolated from leaf litter from fresh water habitat by rehydration technique. Drying also reduce the numbers of gram-negative bacteria, but most actinomycetes have aerial spores that are resistance heat or dessication, thereby, giving chances for isolation of actinomycetes that can produce bioactive metabolites from marine environment (Azman *et al.,* 2015)

New species of *Streptosporangium*, *Microtetraspora*, *Microbispora*, *Actinomadura*, *Thermoactinomyces* and T*hermomonospora* have been isolated using pre-treatment approach such as specialized growth media, long incubation times and dry heating (Kumar and Jadeja, 2016). Also some antibiotics like 50 mg/mL of acetidione, nystatin and 5 mg/mL polymyxin b sulphate has been

incorporated in starch casein medium to selectively grow actinomycetes from soil. Studies revealed that sensitivity of actinomycetes to antibiotics are different, and this has been used to selectively culture actinomycetes. Novobiocin has been used to grow *Thermoactinomycetes* spp., rubomycin and streptomycin for isolation of *Actinomadura* spp. (Kumar and Jadeja, 2016).

In the same vein, some chemicals which can inhibit the growth of bacteria and fungi, such as sodium propionate and phenol have been added into the medium of isolation of actinomycetes so as to reduce the growth of other bacteria and moulds, thereby, favouring the growth of actinomycetes. But these methods usually allow growth of contaminants when the concentration is low and sometimes, hinder the actinomycetes growth when the concentration is too high (Kumar and Jadeja, 2016).

Table 2.2 Selective media for isolation of actinomycetes

Selective agent	Concentration (µg/mL)	Actinomycetes selected

Benzoate	-	*Micromonospora*
Bruneomycin	15 to 35	*Actinomadura*
Gentamicin	-	*Micromonospora*
Kanamycin (25^{o}C)	15 to 25	*Actinomadura*
Kanamycin (50 ^{o}C)	-	*Thermomonospora*
Lincomycin	25	*Micromonospora*
Nalidixic acid	10	*Rhodococcus*
Nitrofurazone	-	*Streptomyces*
Novobiocin (25 ^{o}C)	25	*Micromonospora*
Oxytetracycline	-	*Thermoactinomyces*
Penicillin + NaCl	15	*Streptoverticillium*
Penicillin + polymyxin	15	*Streptomyces*
Polymyxin	5	*Actinomycetes*
Rifampicin (25 ^{o}C)	25	*Actinomycetes*

Source: (Kumar and Jadeja, 2016).

2.1.7. Importance of actinomycetes

Possessing structurally unique secondary metabolites, actinomycetes have become a major source of various potent biological activities. Boonlarppradab *et al.* (2008), reported that major quantity of antibiotics that has been derived from actinomycetes is from the genus *Streptomyces* (Kim *et al.*, 2021)

2.1.7.1 Actinomycetes as producers of different enzymes

Actinomycetes are known to be a good source of antibiotics and other bioactive compounds for many years. Apart from antibiotics are various enzymes. Enzymes from actinomycetes serves various purposes in the industries, such as the textile and pharmaceutical industries, food processing and detergent industries and in medical and molecular biology field (Basha *et al*., 2017). Active strains of *Streptomyces albus* and *Streptomyces chromofuscus* that was isolated from Egyptian soils was reported to produce cellulase free-xylanase. They noticed increase in the enzyme activity of the enzyme when they were both grown on yeast extract, and recorded that five days of fermentation gave the optimum production of the enzyme. Higher bleaching activity of Xylanase was noticed from the one produced from *Streptomyces chromofuscus* when compared with that from *Streptomyces albus*. It enhanced the liberation of reducing sugars, which improved pulp bleachability (Behie *et al.,* 2017).

Kim *et al.* (2003) reported the isolation and purification of chitinase from *Streptomyces* sp. M-20 with optimal activity at pH 5 and 30˚C, and reported stability of about 40˚C from pH 4 to 8 (Huang *et al*., 2017) . It's also showed lysozyme activity against *Botrytis cinerea* cell wall and antifungal activity against *Botrytis cinerea*. Besides, the enzyme activity was completely inhibited by Hg^{2+} and p-chloromercuribenzoic acid. Zarayana and Vijayalakshmi, (2009) reported that under submerged fermentation, the production of chitinase by a terrestrial *Streptomyces* sp. ANU 6277 was promoted by utilization of yeast extract and starch as nitrogen and carbon sources (Mehnaz *et al*., 2017). In a study of mannanase screening in actinomycetes, high levels of the enzyme produced in *Streptomyces scabies* CECT3340 and *S. ipomoea* CECT3341 in liquid culture was reported (Mehnaz *et al*., 2017). The mannanase potential in bleachability improvement of pine kraft pulp was tested. Obviously, it released the colour and chromophoric material from the pine kraft pulp besides increased the pulp brightness. The mannanase production was optimized in *Streptomyces* sp. PG-08-3 from Rajasthan dessert, India was investigated, increment of guar gum concentration in the growth media demonstrated to enhance the production of mannanase (Yamabhai *et al.,* 2016).

Verma *et al*. (2017) reported a first report of two Antartic actinomycetes which produced keratinolytic enzymes to enable their growth on keratin-containing wastes. He reported that *Microbispora aerata* IMBAS-11A (thermophilic) and *Streptomyces flavis* 2BG (mesophilic) demonstrated as very promising strains for effective processing of native keratinous wastes. Tamreihao and Mukherjee, (2019) explained that *Nocardioides, Nonomuraea. Saccharomonospora* and *Streptomyces* are antibiotic producing and keratin-degrading actinomycetes which has the ability to degrade poultry farm feather to biofertilizer that has no pathogen and odour.

2.1.7.2. Biodegradation/bioremediation potential of Actinomycetes

Akansha *et al.* (2019) reported the role of actinomycetes as agent of decolourization in industrial effluents containing water-soluble synthetic reactive dyes like formazan-copper complex, azo-copper complex, phthalocyanine, anthraquinone and Azo dyes. He reported that, actinomycetes displayed Hooda *et al.* (2016), also reported the ability *Streptomyces* spp. to decolourise paper mill effluent obtained after semi chemical alkaline pulping of wheat straw. The highest decolourisation level reported were 60 – 65%.

Different pesticides consisting of chemical structures, such as sulfonylureas, acetanilides, carbamates, s-triazines, organophosphates, triazinones, organochlorines and organophosphonates can also be degraded (Mahanty *et al.,* 2017). Herbicide Diuron has also been reported to be degraded by indigenous soil actinomycetes. It is generally used as weed biocontrol on crops like citrus, cotton, pineapple and sugar-cane at low concentration and also on non crop areas. When Diuron is applied *in vitro,* up to 37% level of herbicide degradation was observed in seven days by the selected actinomycetes.

Actinomycetes uses natural rubber degradation as sole carbon their source, this enable them to be able to degrade rubber (Watcharakul *et al.,* 2016). This was concluded after isolation of variety of rubber-degrading actinomycetes (up to10^5 cfu/g) from soil samples of *Hevea brasiliensis* plantation and from waste water ponds of a rubber-producing company in Malaysia. *Streptomyces, Micromonospora, Actinoplanes, Nocardia, Dactylosporangium* and *Actinomadura* are major rubber degrading actinomycetes. Tandale *et al., (*2018) reported isolation of new *Gordonia* species "*G. polyisoprenivorans"* which is able degrade rubber.

2.1.7.3. Actinomyctes potentials in xenobiotics transformation

The act of structurally modifying components that are stange to the metabolism of an organism is called of xenobiotics Transformation. Condensations, reductive, hydrolytic and oxidative are the most characteristics reactions in xenobiotics transformation. One of the important factors that complicate the process of biodegradation of water and soil pollutants is the potential of actinomycetes to carry out different microbial conversions of organic compounds. *Nocardia* and *Streptomyces* spp has been documented to have the capability to transform complicated compounds of synthetic and natural origin. An aromatic hydrocarbon was degraded by *Nocardia* spp through hydroxylation. They have the ability to degrade some pesticides and herbicides. For example, *Nocardia* strains degraded herbicide; 2, 2-dichloropropionic acid (Dimri *et al*., 2021).

2.1.7.4. Actinomycetes as biosurfactant

Some living cells, especially microorganisms produce some surface-active molecules, called biosurfactant or bioemulsifier, the terms emulsifier and surfactant are mostly used. These compounds are produce by microorganisms that can influence the interface. Biosurfactants can be evaluated through surface tension measurements. Surfactant from microorganisms has some leads over those ones that were synthesize chemically. They can be degraded biologically, less toxic and highly specific. Biosulfactant can be produce from renewable and cheaper feed stocks and also effective when the condition is extremely saline, high pH and temperature. Feller *et al*. (1998) reported bioemulsifiers production (*Trehalose dimycolates*) from *Rhodococcus erythropolis.*

2.1.7.5 Actinomyetes as enzyme inhibitors

Enzyme inhibitor of low molecular weight has been produced from Actinomycetes. Several enzyme inhibitors, such as, Streptonigrin, Revistin, Antipain, leupreptins, they inhibit enzymes like papain, trypsin, chymotrypsin, papain, cathepsnin b and so on. In oncology, enzyme inhibitors are beginning to gain relevancy as they are been used in the treatment of cancer disease. For example, retrostatin and

Streptonigrin that are produced by streptomyces are able to inhibit reverse transcriptase, Alistragin produced from *Streptomyces roseoviridis* are able to inhibits carboxypeptidase b. Phosphoramiden produced by *S. tanashiensi* are able to inhibit metallo-proteases (Bhatti *et al.,* 2017)

2.1.7.6. Antimicrobials and antitumor ability of actinomycetes

Elbendary *et al.* (2018) reported on microbial metabolites production since year 2002, which filamentous actinomycetes produced over 10,000 bioactive compounds (45% of all microbial metabolites). 75% or 7600 of the valuable production were of *Streptomyces* spp. origin and 2500 or 25% were from rare actinomycetes (*Micromonospora, Actinomadura* and *Streptoverticillium*). The approximately proportion of all actinomycetes products demonstrated antimicrobial activity to antitumor activity was 70:30 respectively. Production of a great number of important drugs by actinomycetes is well known. Abhilash, (2015), reported some significant drugs provided by actinomycetes, the likes of aminoglycosides, anthracyclines, chloramphenicol, β-lactams, macrolides and tetracyclines. Among the actinomycetes, *Streptomyces* contributed the greatest chemical diversity (Timkova *et al.,* 2018). A study on obtaining antifungal compounds from marine *Streptomyces* spp. was carried out by Mander *et al.,* (2016). These compounds displayed strong antifungal activity against *C. albicans, E. coli* and *P. aeruginosa*. More recently, Ravitha *et al.* (2017) reported a wide range antimicrobial activity of ten most potent marine *Streptomyces* spp. isolated from the Nahoon beach, a coastal shore of Indian Ocean in the Eastern Cape Province of South Africa. The ethyl acetate extracts of the isolates exhibited activities against atleast 6 and up to 26 of the 32 test bacteria screened. IR spectra analysis was done to characterize the crude extracts and the possibility of terpenoid, long chain fatty acids and secondary amine derivatives compounds in the extracts presence was noticed.

Basically, antitumor compounds are produced naturally mainly by microorganisms. In fact, actinomycete is the major producer of various natural products with different properties including antitumor activity (Malmierca *et al.,* 2018). Two new compounds, spiroaminals, marineosins A and B were produced from *Streptomyces* from marine sediments; they possess significant inhibition of human colon carcinoma (HCT-116) in an *in vitro* and selective activity in diverse cancer cell

types. *Streptomyces* spp. acts as biocontrol agent of fungal pathogens (Bubici *et al.,* 2019). A report by Meij *et al.* (2017) explained an experiment of coating pea seeds with spores/mycelia of *Streptomyces lydicus* WYEC108 to inhibit *Phytium ultimum,* a fungus in an oospore-enriched soil. They discovered that less than 40% of the coated seeds were infected but all uncoated seeds were infected by the fungus 48 hours after planting.

More recently, an Iranian *Streptomyces plicatus* strain 101 was reported having chitinolytic activity and antifungal inhibitory effects on mycelial growth of *Rhizoctonia solani, Sclerotinia sclerotiorum, Fusarium graminearum, F. solani, Rosellinia necatrix* and *Pythium aphanidermatum* mainly by extracellular chitinase production (Altemimi *et al.,* 2017).

2.2. L-asparaginases

2.2.1. Structure of L-asparaginase

Several researchers have elucidated the structure of L-asparaginase. Though it occurs as a tetrameric protein, yet when isolated from different sources, the monomeric, dimeric and hexameric form of it was found. Many of the L-asparaginases from bacterial showed quaternary and tertiary structures (Brumano *et al.,* 2019) *Erwinia* sp. and *E. coli* posses three-dimensional structures which are similar to each other (Chevalier *et al.,* 2017)

L-asparaginase from *Erwinia carotovora* have duo tetramers (ABCD and EFGH), each of them is compose of four similar monomers (A to H). Each monomer consist three hundred twenty-seven amino acids with 14 α-strands, eight β-helices and two domains, a big N-terminal domain and a small C-terminal domain (Qeshmi *et al.,* 2018). The active site is positioned in between two adjacent monomers (A and C: B and D). The tetramer consists of four identical subunits. The whole molecule is considered as a dimer of dimers (Lopez *et al.,* 2017). Every active site is shaped by the conveyance of amino acids in two adjacent monomers. Following amino acids constitute active site: Thr15, Tyr29, Ser62, Glu63, Thr95, Asp 96, Ala120, and Lys168, while only one residue Ser254 is present in adjacent monomer (Li *et al.,* 2019)

2.2.2 Types of L-asparaginases

L-asparaginase has been categorized based on structural, functional and sequence similarities. These are bacterial L-asparaginase, (they are also referred to as type 1 and II L-asparaginase). Plant-type asparaginases, (they are also refered to as type 3 L-asparaginase) and the one that resemble *Rhizobium elti* asparaginase (Brumano *et al*., 2019). Type 1 and II L-asparaginases are in amidase group and make use of threonine as their primary nucleophile during the metabolic reactions. The Type II L-asparaginases are periplasmic, while the type 1 L-asparaginases are cytoplasmic, The Type II posses' maximum affinity to L-asparagine (K_m¼10^5M), and in a little rate aids breaking down of L-glutamine into glutamic acid. *E.coli* L-asparaginase (type 1 and II) are different in their tetrameric structure, but both posses similar conserved amino acid motifs, the type I is known to have low substrate affinity (K_m¼ 10^3M) towards L-asparagine (Brumano *et al*., 2019). Certain strains of yeasts, produces L-asparaginases that contain amino acid sequences similar to the ones in bacterial type II family (Brumano *et al*., 2019).

Plant L-asparaginase posses amino acid sequences which do not the same homology with that of bacterial. They have low affinity to their substrate (as low as K*m*¼10^2M), they do not hydrolyse glutamine. Plant L-asparaginases make use of N-terminal as their primary nucleophile needed in metabolic pathways, in addition with the derivation of nitrogen from the atmosphere. There are two types of plant L-asparaginases, each of them have similar significant levels of sequence. They are, potassium-independent and potassium-dependent. The potassium-dependent L-asparaginase is more found in higher plants and efficiently metabolize L-asparagine when nitrogen is in abundance (Curtis *et al.*, 2018).

L-asparaginase from *Rhizobium etli* are thermolabile, and posses no homology with any other L-asparaginase. Organisms make use of this enzyme when in nitrogen and carbon metabolism (Curtis *et al.*, 2018).

Human genome have the capability to encode three enzymes that can breakdown L-asparagine, they are, lysophospholipase, lysosomal aspartylglucosaminidase and L-asparagine synthase (hASNase3). L-asparagine synthase (hASNase3) resembles plant L-asparaginase and posses a structural homology that resembles that of *E.coli.* Its L-asparaginase activity is dependent on the presence of threonine. Lysosomal aspartylglucosaminidase posses low affinity (K_m¼10^3 M), to it substrate (L-

asparagine) and has up to 60% sequence similarity to that of plant. It helps to breakdown glycoprotein by joining the carbohydrate groups that is attached to L-asparagine. Lysophospholipase breaks down lysophosphatidylcholine including L-asparagine and posses high structural homology similar to that of the one from bacterial (Maggi and Scotti, 2019).

2.2.3. L-asparaginase from microorganisms

Different taxa of organisms have been reported to be L-asparaginase producers, which being produce in huge amount (Sarika and Naveen, 2019). Microbial source L-asparaginase is gaining much relevancy because it is easy to handle, easy to produce in large quantity by submerged fermentation, it can easily be genetically modified, and its production is less tedious and eco-friendly (Sarika and Naveen, 2019). Different groups of microbes like Bacteria. Yeast, Filamentous fungi and Actinomycetes have been reported to produce this enzyme (Qeshmi *et al.,* 2018). Bacteria: *Escherichia coli, Serratia marcescens, Pseudomonas aeruginosa, Pectobacterium carotovorum, Bacillus circulans, Bacillus sp* (Shrivastava *et al*., 2012, Subhash *et al*., 2020). Actinomycetes: *Streptomyces albidoflavus, Streptomyces gulbargensis*, *Streptomyces griseus* (Subhash *et al*., 2020). Fungi: *Fusarium* sp, *Aspergillus terreus*, *Aspergillus niger* (Mihooliya *et al.,* 2020) and *Penicillium sp* (Subhash *et al*., 2020), Cladosporium sp. (Muneer *et al.,* 2020). Yeast: *Mucor hiemalis (*Monica *et al.,* 2013), *Trichoderma viride* (Muneer *et al.,* 2020).

2.2.3.1. Characteristics of L-asparaginases from microorganisms

The efficiency of protein is dependent on different interaction or factors (Subhash *et al*., 2020). Enzyme activity and its effectiveness are greatly affected by pH, temperature, inhibitors and activators, and metal ions. Studies on effect of physical factor and enzymes properties showed the relationship that is present between the environment and the properties of an enzyme, this helps in knowing the factors and activity of an enzyme, to know how to predict and manipulate the protein and to modify the protein structure genetically to perform maximally (Muneer *et al.,* 2020).

2.2.3.2. Effect of pH on the activity and stability of L-asparaginase

From different investigations carried out on the effect of pH on various L-asparaginase activities, optimum pH range from 7 to 8.5 was reported. Optimum pH of L-asparaginase from bacteria was 7.0, while pH 7.5 to 8.5 was observed for

Actinomycetes, and slightly acidic pH 6.2 to 7.5 was observed for fungi (Subhash *et al*., 2020). L-asparaginase at physiological pH is desirable when it's for therapeutic use, and is desirable at acidic pH when use in food processing (Chand *et al*., 2020).

Table 2.3: Effect of varied pH on activity of L-asparaginase

Organisms	Optimum	pH Stability	References
Thermococcu skodakaraensis	9.5		Krishnapura *et al*., 2016
Thermococcus gammatolerans EJ3	8.5		Qeshmi *et al.*, 2018

Erwinia sp.	7–8	No enzyme activity at pH below 4.5	Krishnapura *et al*., 2016
Citrobacter	8–11		Shakambari *et al.* (2019)
Azotobacter vinelandii	8.6		Shakambari *et al.* (2019)
Corynebacterium glutamicum	7.0	Opimum activity was at pH 7.5 and 8.0. Showed no stability at pH 6.0 as it lost all it activity.	Shakambari *et al.* (2019)
Aeromonas sp.	8.25–9.0		
Arthrobacter citreus	8.25–9.0		
Enterobacter aerogenes	8.0		
Thermus thermophilus	9.2		
Erwinia sp.	8.5	Enzyme activity was optimum at pH 8 and 9, and retained it sability at alkaline range	El-Naggar *et al.,* 2018
Pseudomonas aeruginosa	9.0		Fatima *et al.,* (2019)
Pectobacterium carotovorum MTCC 1428	8.49		Souza *et al.,* (2017)
Actinomycetes			
Streptomyces sp. PDK7	8.0–8.5		Shrivastava *et al*., 2016
Streptomyces gulbargensis	7.5	Retained up to 80% activity at pH 8.	Lopez *et al.,* (2017)
Streptomyces noursei MTCC 10469	8		Qeshmi *et al.,* 2018
Fungi			
Cylindrocarpon obtusisporum MB	7.4		Erva *et al.,* (2017)
Aspergillus niger	6.5	Stable between the pH 3.5 to 8.5 there was no loss in the activity o the enzyme under refrigeration.	Batool (2016)
A. niger AK10	8.6	stable at pH 8.0–8.6	Krishnapura *et al*., 2016
Flammulina velutipes	7–8	Stabile between the pH 3 to 9 and retained at least 85% of it activity after 16 hours at the same pH value.	Souza *et al.* (2017)
Penicillium sp.	7.0		El-Gendy *et al*., 2017
Cladosporium sp.	6.3		

Source: (Orabi *et al.,* 2021)

2.2.3.3. Effect of temperature on activity and stability of L-asparaginase

One of the most essential factors affecting the speed of activity and stability of an enzyme is temperature (Nouri *et al.,* 2020, Subhash *et al*., 2020). Temperature has negative effect on stability and activity of an enzyme since at the increase of temperature, there is likely to be an increase in the speed of reaction of the enzyme,

thereby, inactivating the enzyme (Alrumman *et al*., 2019). Therefore, there is need to determine optimum temperature, so as to balance the impact of temperature on the stability and activity of the enzyme. Alrumman *et al*., (2019) recorded optimum temperature of 37^{o}C for L-asparaginases from *Bacillus licheniformis.* Optimum temperature for L-asparaginase from most microorganisms is usually above 30^{o}C (Goswami *et al.,* 2019). L-asparaginase from some Actinomycetes and fungi showed optimum activity at the temperature of 40^{o}C and 45^{o}C (Brumano *et al.,* 2019). Table 2.4 summarizes impact of different ranges of temperature on activity of L-asparaginase.

Table 2.4: Impact of temperature on the activity of L-asparaginase

Organism	Optimum temperature	Temperature stability	References
Archaea *Pyrococcus furiosus*		The activity of enzyme increased with increase in temperature, so optimum	Chohan *et al.,* (2019)

		temperature could not be determined.	
Thermococcus kodakaraensis	85ºC	Thermostable with half-life of 130 minutes at 85ºC.	Chohan *et al.,* (2019)
Thermococcus gammatolerans EJ3	85ºC	75% of relative activity of the enzyme was retained at 70–95ºC.	Qeshmi *et al.,* (2018)
Bacteria			
Azotobacter vinelandii	48ºC	Stable up to 50ºC. At 65ºC, the enzyme became inactive	Shakambari *et al.* (2019)
Corynebacterium glutamicum	40ºC		Shakambari *et al.* (2019)
Aeromonas sp.	40ºC	Enzyme activity was retained at 50ºC, and at 60ºC, 20% of the activity was lost.	Erva *et al.*, 2017
Enterobacter aerogenes		The enzyme ccould not withstand temperatureas total loss of activity was observed.	Erva *et al.*, 2017
T. thermophilus		It was able to withstand temperature, as high as 77ºC.	Batool *et al.,* 2016
Erwinia sp.	35ºC	At 40ºC, the enzyme retained it activity, and lost it activity at a temperature above 60ºC and total loss of activity was observed temperature above 80°C.	El- Naggar (2018)
Pseudomonas aeruginosa 50071	37ºC		Fatima *et al*., (2019)
Escherichia coli	60ºC		Feng *et al.,* (2017)
Pectobacterium carotovorum	40ºC		Feng *et al.,* (2017)
Actinomycetes			
***Marine* Actinomycetes**	50ºC		Lopez *et al.,* (2017)
Streptomyces gulbargensis	40ºC	55% of the enzyme activity was retained at 80°C	Brumano *et al.,* (2019)
Streptomyces noursei MTCC 10469	50ºC		Qeshmi *et al*., (2018)
	37ºC		Erva *et al.,* (2017)
Fungi			
Cylindrocarpon obtusisporum MB-10			
Candida utilis		At 50ºC, the enzyme showed moderate stability and become inactive at this temperature.	Chand *et al.,* (2020)
Aspergillus niger	40°C	At 60°C, the enzyme retained 50% of it activity.	Batool *et al.,* (2016)
Penicillium sp.	37°C	Quite stable at high temperature.	Gendy *et al*., (2017)
Cladosporium sp.	30ºC		Muneer *et al.,* (2020)

Source: (Orabi *et al.,* 2021)

2.2.3.4. Influence of effector molecules on the activity and stability of L-asparaginase

The reactions of inorganic and organic molecules lead to chemical modifications that determine the chemical property and stability of an enzyme (Krishnapura *et al*., 2016). To better understand the enzyme characteristics and its mechanism of action, It is expedient to study various effectors that inhibit or activate the enzyme and it activity. These aid to tailor the enzyme to fit into biotechnology purpose in which it is needed and to increase the enzyme's catalytic efficiency. In several occasions, availability of effectors is significant in stabilizing the reaction intermediate of an enzyme, and when the multimeric structure of an enzyme is to be maintained. Impacts of different effectors on activity of L-asparaginase activity is illustrated in Table 2.5. Activity of *Erwinia carotovora* and *Cylindrocarpon obtusisporum* MB-10 L-asparaginase was enhanced by EDTA (Qeshmi *et al.,* 2018), on the contrary, L-asparaginase activity from *Pectobacterium carotovorum* MTCC 1428 and *Cladosporium* sp. was not affected by EDTA (Radha *et al.,* 2018, Muneer *et al*., 2020). When an enzyme is inhibited by EDTA, it means that enzyme is metaloprotein. Also, The activity of L-asparaginase from *Azotobacter vinelandii*, *Pectobacterium carotovorum* MTCC 1428, *Erwinia carotovorum* and *Cylindrocarpon obtusisporum* MB-10 was inhibited in the presence Cd^{2+}, Zn^{2+} and Hg^{2+}, and this implies the existence of essential vicinal sulfhydryl group(s) (Qeshmi *et al.,* 2018, Muneer *et al*., 2020).

Also, increase in the activity of an enzyme in the presence of thiolprotecting agents like reduced glutathione, dithiothreitol and 2-mercaptoethanol, reduction with thiol group blocking reagents, like, iodiacetamide and p-chloromercuribenzoate gave an evidence that sulfhydryl groups helps in maintaining the catalytic activity and in suitable conformation of these enzymes. During the study of *Erwinia carotovora* L-asparaginase kinetic parameters using agents like L-methionine, N-acetyl cysteine, reduced glutathione and L-cystine, increased and decreased in the V_{max} and K_m values was observed respectively. This indicates a non-essential mode of enzyme activation (Qeshmi *et al.,* 2018). Phenyl–methyl–sulfonyl fluoride inhibited L-asparaginase from *Cladosporium* sp. this indicated that serine was involve in the active site. Muneer *et al*., (2020) observed increase in the activity of an enzyme when N-bromosuccinimide.is present Also, when L-asparaginase from *Thermus thermophilus* was chemically modified by Radha *et al*. (2018), the presence of carboxylic residues, arginyl and histidyl were observed at or near the

active site, whereas, cysteine and serine were required for the active form of the enzyme.

Table 2.5: Impact of different molecules on activity of L-asparaginase.

Organisms	Activators / enhancers	Inactivators/ depresser	Effectors with no effect	References
Archaea *Thermococcus kodakaraensis*			EDTA , Mn^{2+}, Ca^{2+}, Cu^{2+} Zn^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, and Cd^{2+},	Chohan and Rashid (2019)
Thermococcus gammatolerans EJ3	Mg^{2+}	Cu^{2+}, Ba^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}		Qeshmi *et al.*, (2018)
Bacteria *Azotobacter vinelandii*		Hg^{2+}, $Cu^{2+}$and Zn^{2+}, Acted as inhibitors based on their concentrations.	Amino and keto acids	Qeshmi *et al.*, (2018)
Corynebacterium glutamicum			20 natural amino acids including some monovalent and divalent cation, such as K^{+} and Mg^{2+}.	Shakambari *et al.* (2019)
Aeromonas sp. .		Hg^{2+}, Cu^{2+} and Fe^{3+}	Sodium, potassium, calcium and magnesium	Erva *et al.,* (2017)
Thermus thermophilus		Zn^{2+} partially inhibits	Divalent metals – $Mg^{2+,}$ Mn^{2+}, Ca^{2+}	Batool *et al.*, 2016
Erwinia sp.		EDTA	Metal ions	El- Naggar *et al.,* (2018)
Erwinia carotovora	Na^{+}, K^{+} and EDTA	Zn^{2+}, Mg^{2+}, Cd^{2+}, Fe^{3+}, Cu^{2+}, Hg^{2+}, and Ni^{2+}.	L-histidine and L-cysteine. No effect was observed from other amino acid.	Qeshmi *et al.,* (2018)
Bacillus sp.	$MgCl_2$	EDTA		Orabi *et al.,* (2020)
Pectobacterium carotovorum MTCC 1428	Glutathione, 2-mercaptoethanol K^{+}, Na^{+}, L-histidine and L-cystine.	Various thiol group blocking reagents and divalent cations moderately inhibited it.	EDTA	Feng *et al.,* (2017)
Actinomycetes *Marine actinomycete*	Mg^{2+}	Cu^{2+}, Zn^{2+} and EDTA		Lopez *et al.,* (2017)
Fungi *Cylindrocarpon obtusisporum MB-10*	Cysteine reduced glutathione and EDTA.	Cu^{2+}, Ni^{2+}, Fe^{2+}, Zn^{2+} and Hg^{2+}.		Erva *et al.,* (2017)
Cladosporium sp.	Fe^{3+}, Pb^{2+}, KI and detergents	$Mg^{2+,}$ Cu^{2+} , SDS, Ca^{2+}, Co^{2+}.	EDTA	Muneer *et al.,* (2020)

Source: (Orabi *et al.,* 2019)

2.2.3.5. Molecular weight of L-asparaginase enzyme

The chemical complexity, homogeneity and molecular weight of foreign macromolecule is large, and is proportional to it immunogenicity. Huang *et al.,* (2020) reported that, if molecular weight of a compound is greater than 6 kDa, it will be immunogenic. A specific antibody was formed in patients treated with L-asparaginase that has larger molecular weight of about 140 kDa (El-Fakharany *et al*., 2020). The differences in molecular weights of L-asparaginases from different microorganisms are much; as recorded in Table 2.6.

Table 2.6: L-asparaginases molecular weight from different microorganisms.

Organisms	Molecular weight	References
Archaea		
Pyrococcus furiosus	37 kDa	Chohan and Rashid (2019)
Thermococcus kodakaraensis	35.5 kDa	Chohan and Rashid (2019)
Thermococcus gammatolerans EJ3	74 kDa	Qeshmi *et al.,* (2018)
Bacteria		
Citrobacter sp.	166 kDa	Shakambari *et al.* (2019)
Vibrio succinogenes	146 kDa	Ebrahimipour *et al.,* (2020)
Azotobacter vinelandii	Approximately 84 kDa	Shakambari *et al.* (2019)
Enterobacter aerogenes	46 kDa.	Muneer *et al.,* (2020)
T. thermophilus	33 kDa per subunit	Batool *et al.,* (2016)
Pseudomonas aeruginosa	160 kDa	Fatima *et al.,* (2019)
Bacillus circulans	140 kDa	Abdelrazek *et al.,* (2020)
Erwinia carotovora	Approximate molecular mass of 150 kDa	Qeshmi *et al.,* 2018
Pectobacterium carotovorum	144.42 kDa.	Feng *et al.,* (2017)
E. chrysanthemi	140 kDa	Silva *et al.,* (2020)
Actinomycetes		
Streptomyces gulbargensis	85 kDa	Brumano *et al.,* (2019)
Streptomyces noursei MTCC 10469	102 kDa	Qeshmi *et al.,* 2018
Fungi		
Flammulina velutipes	85 kDa	Souza *et al.,* (2017)
Penicillium sp.	66 kDa	Gendy *et al.,* (20172)

Cladosporium sp.	120 kDa	Muneer *et al.,* (2020)

Source: (Orabi *et al.,* 2021)

2.2.3.6. Substrate affinity of the enzyme (K_m)

The relative affinity between the enzyme and its substrate largely depends on affinity of the enzyme to its substrate 'Michaelis constant K_m' (Muneer *et al*., 2020). Sally *et al.* (2020) stated that the variations in antitumor properties of L-asparaginase might be trace to substrate affinity of the enzyme. Robert *et al*. (2020) mentioned that, degree of effectiveness of L-asparaginase against tumors cells is related to affinity to its substrate. Different microorganisms have different K_m values which range from 0.01mM to 12mM. Organisms like *Citrobacter freundii*, *Vibrio succinogenes, Erwinia carotovora and E. coli produces L-asparaginase with* low K_m values (0.01 to 0.05mM), this enzyme has high substrate affinity, which makes them to be highly potent against tumor cells (Goswami *et al.,* 2019). In another shell, high K_m value of 10.07mM was observed from L-asparaginase from *Streptomyces fradiae* and yet has anticancer property (El-naggar *et al.,* 2019).

Table 2.7: Kinetic properties of microbial source L-asparaginases

Organism	K_m	References
Bacteria		
Thermococcus kodakaraensis	5.5mM	Chohan and Rashid (2019)
Thermococcus gammatolerans EJ3	10mM	Qeshmi *et al.*, (2018)
Bacillus megaterium (H-1)	0.8mM	Shamkabari *et al.,* (2019)
Escherichia coli (DE3)	1.2mM	Muneer *et al.,* (2020)
Bacillus subtilis B11—06	0.043mM	Balbool *et al.,* (2020)
Staphylococcus sp. (OJ82)	2.2mM	Sahoo and Sahooo, (2021)
		Guo *et al*., (2017)
Corynebacterium glutamicum	2.5mM	Shakambari *et al.,* (2020)
Aeromonas sp.	0.0175mM	Erva *et al.,* 2017
Enterobacter aerogenes	1.25mM	Muneer *et al.*, (2020)
Thermus thermophilus	2.8mM	Batool *et al*., 2016(2001)
*Erwinase_*	0.09605mM	Chand *et al.*, (2020)
Kidrolase	0.03306mM	Chand *et al.*, (2020)
Erwinia sp.	0.22mM	El-Naggar (2018)
Pseudomonas aeruginosa 50071	0.147mM	Fatima *et al.,* (2019)
Erwinia carotovora	0.098mM	Qeshmi *et al.,* (2018)
Pectobacterium carotovorumMTCC 1428	0.657mM	Feng *et al.,* (2017)
Actinomycetes		
Thermoactinomyces vulgaris13 M.E.S.	4.5mM	Silpa *et al.,* (2017)
Marine actinomycete	0.024mM	Lopez *et al.,* (2017)
Fungi		
Cylindrocarpon obtusisporum MB-10	1mM	Erva *et al.,* (2017)
Penicillium sp.	4mM	Gendy *et al*., 2017
Cladosporium sp.	100mM	Muneer *et al*., 2020

Source: (Faizan *et al*., 2020)

2.2.4. Anticancer activity of L-asparaginase

Clementi (1922) reported that, active L-asparaginase was present in blood serum of some herbivores and omnivores, but not present in the blood serum of carnivores, reptiles and Amphibian (Balbool and Abdel-Azeem, 2020). Kidd (1953) treated lymphomas in mice and rats with Guinea blood serum, and observed a regression in the disease. Broome (1963) reported that, Guinea pig serum has L-asparaginase, which makes it to have anti-neoplastic activity. Later on, some researchers established that purified L-asparaginase from Guinea pig has antitumor activity (Yim and Kim, 2019). Anticancer drug was then discovered from these discoveries. The needs for more L-asparaginase as drugs led to sourcing for this enzyme among microbes (Yim and Kim, 2019), which are real source of diverse secondary metabolites. *Escherichia coli* was screened for L-asparaginase production, it was discovered that L-asparaginase produce from it has anti-neoplastic activity, which is closely related to that from Guinea pig serum **(**Orabi *et al.,* 2019). L-asparaginase produced by *Escherichia coli* are of two types; type I and II, Type II have been said to have high substrate affinity, which makes it to have more antitumor effect. The discovery of L-asparaginase with antitumor property from some enteobacteriaceae sprouts up significant interest in screening and production of L-asparaginase from diverse microorganisms (Faizan *et al*., 2020). L-asparaginase from microbial sources can be produce in large scale, which makes it more available in the industrial and pharmaceutical industries (Faizan *et al*., 2020).

Of all the diagnosed cancers in children between ages 0–14 years, leukemia accounts for one-third of it (Saeed *et al.,* 2020). The introduction of L-asparaginase into the treatment of ALL was since 1970s (Zenatti, *et al.,* 2018) and the success of this treatment is singly or in support with some well documented chemotheraupeutic drugs. Several studies have documented that L-asparaginase has cytotoxicity effect against some cancer cell including leukemic cells (Costa-Silva *et al.,* 2020). L-asparaginase has been used in multiagent chemotherapy against Hodgkin lymphomas (HL, non-Hodgkin lymphomas (NHL) and Natural Killer (NK)/T-cell lymphoma which has yielded good result (Krishnapura *et al.,* 2016). When a tumor cell is resistance to chemotherapy, L-asparaginase could be

an alternative to reduce it growth; this is due to the fact that the antitumoral mechanism of L-asparaginase is original, so that multi-drug resistance cannot affect it (Hu *et al.,* 2020). Up to date, L-asparaginase is included in the treatment for the remission of ALL and it can be used at any stage (Krishnapura *et al.,* 2016).

Asparaginase Medac, Leunase, Kidrolase and Elspar are brand names given to L-asparaginase produced from *Escherichia coli* is the pharmaceutical industry (Saeed *et al.,* 2020). FDA, in July 2006 approved a a pegylated form of *E. coli* asparaginase called Oncaspar (Beckett and Gervais, 2019). Erwinase, an L-asparaginase produced from *Erwinia chrysanthemi* was approved for usage in the treatment of ALL in November 2011 (Chakraborty and Shivakumar, 2021). Currently, a pegylated form of recombinant *Erwinia* asparaginase and recombinant *Escherichia coli* asparaginase are under preclinical studies (Saeed *et al.,* 2020).

2.2.5. Uses of L-asparaginase in food industry

Attention of food scientist has been drawn to the efficacy effect of L-asparaginase in limiting the formation of a carcinogenic compound called acrylamide in food processing. Acrylamide (2 propenamide), is colorless, and odourless crystalline solid, which is synthesize when food containing reducing carbonhydrate and L-asparagine are heated at temperature above 120^{o}C, due to maillard reactions (Muneer *et al.,* 2020).

Acrylamide formation in variety of stachy food, weather oven cooked or fried food has been documented by several researchers, French fries and potato Chips (Lincoln *et al*., 2021) bakery product (Orabi *et al*. , 2021) are cooked at high temperature and they have been a suspected sources of acrylamide (Maan *et al.* 2020). Several studies reported the presence of acrylamide in fried or baked food; this led to the introduction of L-asparaginase in food industries (Parisi and Luo, 2018). Maan *et al.* (2020), reported that up to 0.3kg to 0.6kg of acrylamide is being ingested by adult per day, mean while adolescent and children tends to ingest more daily. Acrylamide has been termed as ‘carcinogenic compound to human” by the International Agency for Research on Cancer (IARC, 1994) based on several studies (Corrêa *et al*., 2021). European Commission Scientific Committee on Food (2002) attested to the intrinsic toxic effects of acrylamide (carcinogenicity, reproductive toxicity, neurotoxicity and genotoxicity) to both somatic and germ cells (Orabi *et al.,* 2021)

From current studies, it was observed that most breast, ovarian, endometrial cancer and renal dysfunctions risks have a positive association with acrylamide intake (Mollakhalili-Meybodi *et al.,* 2021). Genotoxicity and carcinogenicity effect of acrylamide was reported to be due to glycidamide formation (Mollakhalili-Meybodi *et al.,* 2021), in that cytochrome P450 mechanism in humans convert acrylamide to glycidamide, which later forms DNA-glycidamide adducts and haemoglobin adducts, which cause genetic damage (Horiuchi *et al.,* 2021). Degradation of L-asparagine in the presence of heat can leads to acrylamide (Mollakhalili-Meybodi *et al.,* 2021). Muneer *et al.* (2020) reported that the main precursor for acrylamide formation is free L-asparagine, which play more roles than other factors, including reducing sugars.

Reducing acylamide content in food should be done without affecting the sensory and nutritional quality of the food (Liyanage *et al.,* 2021). Several suggestion has been made in reducing the acrylamide levels in foods, such as, additions of antioxidants (Schouten and Tappi, 2021), allicins, filamentous fungi for example, *A.oryzae* and through agronomic and genetic approach (Zilic *et al.,* 2020). On the other hand, using additives in reducing acrylamide level may have some unwanted effect on the sensory and nutitional value of the food, thereby, making L-asparaginase more important and relevant in food industries (Zilic *et al.,* 2020).

Several researchers suggested that appropriate solution to reducing L-asparagine levels in food or reduced the risk of acrylamide production in food is pretreatment with L-asparaginase. A 88% reduction of L-asparagine which correspond to 99% acrylamide reduction in the final cooked product was reported by Bertuzzi *et al.* (2020) when the potato was treated with L-asparaginase before cooking. 70% reduction in the acrylamide content was attained when L-asparaginase was applied to cracker product, during dough preparation (Bertuzzi *et al.* (2020). Pedreschi *et al.* (2004) observed that when L-asparaginase was applied to potato to remove L-asparagine and also reduce acrylamide formation, the enzyme performed efficiently to accomplished the reduction of acrylamide in French fries (Liyanage *et al.,* 2021). Corrêa *et al.* (2009) used L-asparaginase that are cloned and expressed in *Aspergillus oryzae*, on foods, such as, crisp bread, ginger biscuits, French fries, sliced potato chips and semisweet biscuits, and in dough based applications, 34–92% acrylamide reduction was observed in the final products. Drastic reduction of

about 80% in acrylamide content of fried potato strips immerse in L-aspraginase was reported by Chand *et al.* (2020). Muneer *et al*. (2013) used *Cladosporium* sp. L-asparaginase to treat fried potato and achieved reduction of about 96% in acrylamide formation. Raveendran *et al*. (2018) reported that traeatment of dough with *Cladosporium* sp. L-asparaginase worked perfectly not only in reducing acrylamide content, but reduced hydroxymethylfurfural formation, agenotoxic intermediate of Maillard reactions. Currently, duo product of L-asparaginase are commercially available in the market, they are, preventase and Acrylaway (Bruno *et al.,* 2019).

2.2.6 Uses of L-asparaginase in biosensors development

Biosensor that is based on L-asparaginase is used to detect the level of L-asparagine in blood serum of leukemia patient. This is done by different techniques of enzyme mobilization, like entrapping L-asparaginase between the ammonia gas permeable membrane and cellophane dialysis membrane (Doriya *et al*., 2018). Biosensor can measure the L-asparaginase level in blood serum, ranging between 10^{-10} and 10^{-1} M (Doriya *et al*., 2018). Balbool and Abdel-Azeem (2020) reported that biosensor based on L-asparaginase can detect 10–9 M level of L-asparagine.

L-asparaginase can be co-immobilized using the indicator (penol red) on calcium alginate beads, nitrocellulose membrane and silicone gel to develop a biosensor which can be use as diagnostic tools to detect L-asparagine in leukemia and normal serum samples using color visualization. Co-immobilized L-asparaginase producing microbes and phenol red Balbool and Abdel-Azeem, (2020) indicator with tetramethyl orthosilicate gel, to develop a whole-cell based fiber optic biosensor. This is also useful in food industries for monitoring L-asparagine content in food samples.

2.2.7. Challenges encountered in reducing cancer cells using L-asparaginases

Several microorganisms produce L-asparaginase, but L-asparaginase from *Erwinia sp* and *E.coli* are sufficient and used in the treatment of acute lymphoma leukemia and some human malignancy that depends on L-asparagine for survival**.** There are several adverse effect that has developed from the medical use of L-asparaginase prepared from *Erwinia chrysanthemi* and *Escherichia coli*, which act as a threat to life (Jameel *et al.,* 2020), this includes, formation of anti-asparaginase antibodies,

drug resistance, anaphylactic shock and sometimes, neutralization of drug, to mention but a few (lima *et al.,* 2020)

To mitigate the side effect from L-asparaginase in the case of toxicity and hypersensitivity in patient, L-asparaginase which has a different pharmacological property can be substituted with the one used in the first-line therapy (Ekpenyong *et al.*, 2021). That is, changing L-asparaginase that causes side effect in one patient to the one from another source. For example, changing L-asparaginase from *E.coli*, that causes hypersensitivity reaction in a patient, to that from *Erwinia sp*. or PEGL-asparaginase, thereby, reducing the side effect (Lima *et al.,* 2020), though the substituted ones too, also have side effect, this is due to interactions between the antibodies directed against the L-asparaginase from these sources (Ekpenyong *et al.,* 2021). Balbool and Abdel-Azeem, (2020) reported, there is no difference between the adverse effect initiated by *Escherichia coli* and *Erwinia chrysanthemi* L-asparaginase, PEG asparaginase have more advantages over the others, which is very obvious, yet it silent inactivation, toxicity cannot be neglected (Ghasemi *et al.,* 2017). The occurrence of anti-PEG-asparaginase antibodies has been reported by Vimal and Kumar, (2020) which eradicate the enzyme faster and make the treatment not to be effective. Some researchers reported that, Belen *et al.* (2020) when given PEG-L-asparaginase in a multilevel treatment, its toxicity seem to be like that of other L-aspraginase used in frontline treatment. Which means, PEG-L-asparaginase cannot be always used in the stead of other L-asparaginase as it is also limited to be used therapeutically (Akgun *et al.,* 2021).

Due to the earlier-mentioned limitations with the available L-asparaginase, there is a need for newer L-asparaginase that is different in their antigenic properties and also useful in re-induction therapy. 20–25% of the patients as reported by El-Fakharany *et al*. (2020) are likely not to re-experience allergic reactions when exchange non-cross reactive L-asparaginase preparation, and recorded that 90% of the patients completed their treatment. Hijiya and Sluis, (2016) state that, if this preparation is used for both induction and reinduction therapy, it will make the rate of immunological reactions to be high. This depicts that, other than the already available L-asparaginase, newer L-asparaginase can be making use of in re-induction treatment. Also, in the pharmaceutical industry, the low half-life and stability of L-asparaginase is more of concern (Balbool and Abdel-Azeem, 2020).

The need for multiple doses, which trigger hypersensitivity reactions, can be prevented, when the enzyme has increased half-life and is highly stable. The immunological, clinical and genetic characteristics also influence the efficiency and result of treatment of cancer with L-asparaginase (Paul and Tiwary, 2020). If much quantities of newer L-asparaginase are available, which have different antigenic properties; it can lead to comprehensive study on the immunogenicity, activity, structure and anti-cancer properties of varied L-asparaginases, thereby, leading to new drug development and give more knowledge on individual treatment.

2.2.8. Reasons for L-asparaginase as tools in food processing

L-asparaginase breaks down L-asparagine, which makes it a likely tool in the reduction of quantity of free L-asparagine in starchy food, thereby, decreasing the likelihood of production of acrylamide, (neurotoxin and carcinogenic compound). In food industries, sophistication of food processing is on the high side, which gives a great request for different types of enzymes that have features that are in line with processing of food guidelines (Jemli *et al.,* 2016).

2.2.9. Ways to overcome L-asparaginase challenges

2.2.9.1 Molecular modification of L-asparaginase

Diverse effort has been put into chemical and molecular modification of protein, so as to ensure enhanced specific activity and stability, and to decrease immunological response from it. In engineering an enzyme, that is, protein engineering, an amino acid sequence of an enzyme is altered by bio-molecular methods, like strengthening its structure by mutating a specific site or directed evolution (Brumano *et al.,* 2019). Several submissions has been made on modification of L-asparaginase using these methods that has yielded in improved kinetic properties, longer half-life, large pH and temperature-range for the activity of the enzyme, enhanced resistance to proteolytic digestion and higher thermostability and specific activity. dextran, polyethylene glycol (PEG), polyvinyl alcohol, and albumin are soluble polymes involved in chemical modification of L-asparaginase and insoluble support matrices like polyacrylamide gels, collagen and carboxymethyl cellulose has also been reported (William *et al.,* 2020).

The covalent joining of PEG with L-asparaginase is the general method for the modification of L-asparaginase. Though this method is effective alternative to

Erwinia sp and *Escherichia coli,* yet, its side effect cannot be underestimated, such as, toxicity and loss of activity. This makes researchers to study on new method of enzyme modification for L-asparaginase improvement.

Chemical modification of *Cladosporium* sp. L-asparaginase with bovine serum albumin and ovalbumin increased the stability and activity of the enzyme (Muneer *et al.*, 2020). The antigenecity of *Erwinia chrysanthemi* L-asparaginase was reduced when site-directed mutagenesis was used by Sigh *et al.* (2020), this is done by using threonine to replace the residues of proline (essential for antibodies binding). Vidya *et al.* (2016), change the surface charges on L-asparaginase from *E.coli* by directing mutation to aspecific site and exchange destabilized amino acids with stable residues to achieved enhanced stability (Sahoo and Sahoo, 2021).

Another method that is used, which can improve bioreactor operation, impart stability and prevent loss of activity, is enzyme immobilization. L-asparaginase from *E.coli* was covalently immobilized to achieve multimeric structure that is a fully stable, this deprived subunit dissociation to inactivate the enzyme.

William *et al.* (2020) immobilized L-asparaginase using glutaraldehyde. Higher substrate affinity (times eight) of the immobilized enzyme than the native L-asparaginase was achieved.Tarhan *et al.* (2020) used polyaniline nanofibers to immobilized L-asparaginase and recorded improved activity.

L-asparaginase can be protected against proteolysis by trypsin by the aid of (MABs) 'non-inhibitory monoclonal antibodies' as reported by Krishnapura *et al.* (2016). Also, using red blood cells as biodegradable and biocompatible tools has been suggested so as to limit the re-occurrence of adverse effects and enhance enzyme efficacy (Prakash *et al.,* 2020).

2.2.10. Development of new L-asparaginases

Thorough studies have been done on modification of L-aparaginase from *Escherichia coli* and *Erwinia* sp. Going further to explore the world of microbes that consist of enzymes with different functional and structural properties could be a means of getting L-asparaginases that have features that fits their use in

biomedical fields, and developing a solutions to L-asparaginase enzyme modification strategies. Though, diverse microorganisms have the ability to produce L-asparaginase, yet, there is variation in the properties of this enzyme depending upon the microbial source. The serological, physiological and pharmacological properties of L-asparaginases isolated from different microbial sources are different (Prakash *et al.,* 2020). The activity, efficacy and pharmacokinetic properties of L- asparaginases from different biological sources vary, so also, their ability to induce side effect (Hernández-Marqués *et al.,* 2020). Disparities in the bio-activity and half-life of enzymes generated differently have been reported through pharmacokinetic studies. Pharmacokinetic differences between individual *E. coli* preparations and that of *Erwinia* and *E. coli* L-asparaginases have been reported (Hatamzadeh *et al.,* 2020).

Microbial source L-asparaginase varied in terms of their properties like molecular weight, K_m values and their response to effectors molecules. L-asparaginase isolated from different microorganisms other than *Erwinia sp* and *Escherichia coli* are likely to have much activity with fewer side effects (Muneer *et al.,* 2020)

2.2.11 Prospective source of L-asparaginases (Endophytes)

Microbes that live in the internal tissues of plants without posing any threat or symptoms of disease are called endophytes; they include actinomycetes, fungi and bacteria (Wardah *et al.,* 2021). They are new sources of different compounds that are pharmaceutically important (Shukla *et al.,* 2021) especially the ones from medicinal plants have been discovered that they have ability to produce metabolite that can be used to develop new compound (Balbool and Abdel-Azeem, 2020). Endophytes produce different natural compounds that have special structures and enormous bioactivities, which make them a large reference for industrial, medicinal and agricultural uses (Huang *et al.,* 2020).

There is no much attempt in isolating endophyte that can produce L-asparaginase, neither is there any data about the catalytic mechanism and molecular structure of L-asparaginase from endophytes. *Colletotrichum* sp. E1T9 (an endophyte) was isolated by Chow and Ting, 2021 from *Hiptage benghalensis* (a medicinal plant) and showed high potential for L-sparaginase production with maximum and moderate activity against colon cancer (CaCO2) and hepatocyte carcinoma (HepG2) cells line respectively in vitro (Chow and Ting, 2021). Three L-asparaginase

producing endophytic bacteria was isolated by Tandon *et al.* (2010) using agar-plate method. Sixty-four endophytic fungi with L-asparaginase ability was isolated from was isolated from marine algae by Thirunavukkarasu *et al.* (2021), out of which the highest L-asparaginase production was recorded from *Fusarium* sp. isolated from *Sargassum wightii thallus*. Twelve strains of filamentous fungi that can produce L-asparaginase were isolated from the leaves and bark of *Aegle marmelos Linn* (Hatamzadeh *et al.,* 2020). Twenty-five isolates tht can produce L-asparaginase was isolated from *Pereskia bleo, Murraya koenigii, Cymbopogon citrates and Oldenlandia diffusa* (Chow and Ting 2021). Few endophytic fungi that can produce L-asparaginase have been reported to be isolated by Bhavana *et al.* (2020) from a plant *Tabernaemontana heyneana.*

2.12. Other sources of L-asparaginase

Microorganisms from marine environment have also been reported to have high ability for L-asparaginase production (Alzahrani *et al.,* 2020), due to the high concentration of salt in marine habitat, it make it possible for enzymes from this source to be able to tolerate salt. It has also been reported that microalgae *Chlamydomonas* spp have the ability to produce L-asparaginase (Wardah *et al.,* 2021). Actinomycetes *Streptomyces noursei* MTCC 10469 isolated from marine sponge *Callyspongia diffusa* have been reported to be L-asparaginase producer (Muneer *et al.,* 2020). Production of L-asparaginase from microorganisms isolated from fishes and prawns has also been reported (Ekpenyong *et al.,* 2021).

2.3. Acrylamide

Cooking of foods has numerous advantages including destruction of microorganisms, elimination of heat-sensitive toxins, increase in nutrient bioavailability, and development of desired color, flavor, and texture. Heating may be also responsible for the formation of toxicants, among which are acrylamide.

Great attention has been directed toward the possible undesired effects of acrylamide. Acrylamide (C_3H_5NO) also known as prop-2-enamide is a white odourless crystalline solid. It is soluble in chloroform, ethanol, ether and water. In the presence of bases, acids, iron, iron salts and oxidizing agents, it decomposes non-thermally to form ammonia, and produces carbon dioxide, carbon monoxide and oxides of nitrogen in thermal decomposition (NOC, 2014). Acrylamide has been termed as a potential carcinogenic and toxigenic compound, and can be in uncooked or raw food naturally in minute concentration. This compound must be in large quantity before it can cause any toxigenic or carcinogenic effect. It can be in larger in food when its been cooked. Other means of exposure to this compound are non dietary, like use of cosmetics, use of cigarette and also airborne release of this compound in the manufacturing industries producing products like, petroleum, photographic film, paper, dyes, construction adhesives and asphalt (NOC, 2014).

2.3.1 Chemical characteristics of acrylamide

Acrylamide has a high boiling point around 136^{o}C, with a melting point of 84.5±0.3°C, molecular weight of 71.08 and low vapour pressure of 0.14 at 55°C (Li *et al.,* 2020). Acrylamide is soluble in some compounds and not soluble in some, it depends on their solubility. It has high solubility in water. Acrylamide contain an amide group, reactive electrophilic bond and it is a dysfunctional monomer, did not have a chromophore that is strong enough to detect UV and lack ability to fluorescence. It has basic and weakly acidic properties (Li *et al.,* 2020).

2.3.2 Toxicity of Acrylamide

The toxigenic effect of acrylamide has been established and it has the capacity of causing diverse toxic effect, such as human neurotoxic effect (Meghavarnam and Janakiraman, 2018). It has been termed as carcinogen in human (Li *et al.,* 2020). This is due to glycidamide (epoxy metabolite)

2.3.3 Occurrence of Acrylamide

Acrylamide is been used in paper and pulp industry, textile processing industries, in waste and water treatment, mineral and concrete processing, crude oil production processing, in sand and soil treatment, in cosmetics, photographic emulsion, coat application. It can also be generated from smoking, but not seen as a challenge in making tobacco. Polymers of acrylamide has been widely use in

several industrial processing, such as, textile processing, concrete processing, water and waste-water treatment, soil and sand treatment, paper and paper pulp processing, mineral processing, crude oil production processes. They are also applied as additives to cosmetic, photographic emulsion (Li *et al.,* 2020). It is also formed in tobacco smoke, though not identified as a challenge in smoking tobacco (Li *et al.,* 2020).

2.3.4 Acrylamide in foods

Different organisms have been reported have exposed to acrylamide, such as, free living organisms, cattle and human. Humans are more exposing to it which corresponds to it high level in human. Tracing the sources of exposure to acrylamide, due to cancer risk accorded to it, it was from heating of food at a high temperature above 120^{o}C (Meghavarnam and Janakiraman, 2018). When different food was heated independently using microwave by heating or frying pan by frying, acrylamide was formed with high amount in product of potato.

The formation of acrylamide in food is in two ways, it can be due to interaction of sugar and amino acid when heating. Various kinds of sugars and several amino acids can react likewise, yet L-asparagine has a greater ability to react with sugars to form acrylamide at high temperature than other amino acids due to maillard reaction (Li *et al*., 2020). It can also be due interaction between the L-asparagine and sugar carbonyl group. This byproduct due to maillard reaction is undesirable and occurs usually in food with low moisture when heated e.g. roasted coffee, bakery product and different derivative of potatoes.

Nevertheless, acrylamide can be formed in the absence of sugar; it can be due to oxidation of fat in food which formed 3-carbon molecules (including acrylic acid and acrolein), and these 3-carbon molecules react with L-asparagine in the presence of heat to form acrylamide. This formation of acrylamide usually occurs in fried food, where little sugar is present and no addition of sugar and minute catalysis of starch to sugar occurred (Li *et al.,* 2020).

Table 2.8: Different amino acids contributes to acrylamide formation involving sugar and heat

Amino Acid*	Level of Acrylamide Formation After Combination with Sugar and Application of Heat
Alanine	<50 ppb
L-asparagine	9270 ppb

Aspartic Acid	<50 ppb
Cysteine	<50 ppb
Glutamine	156 ppb
Lysine	<50 ppb
Methionine	<50 ppb
Threonine	<50 ppb

Source: (Palus *et al.*, 2018).

2.3.5 Factors affecting acrylamide content

One of the factors that influence acrylamide formation is time and temperature. Acrylamide cannot be formed at temperature lower than 120^{o}C that is needed for it formation. Which means it cannot be formed while boiling (Elballat *et al.*, 2020). The result of the outcome of the formation of acrylamide and it diasappearance, that is, net formation is what is usually analyzed in food. When the cooking is extended at higher temperature, there will be decrease in the formation of

acrylamide. Observation has been recorded that long time of heating at high temperature reduce the net formation of acrylamide (Elballat *et al.*, 2020).

pH is another factor that influence the acrylamide formation, browning reactions occurs at pH 8, where acrylamide is formed. The maximum acrylamide formation was at pH 8, when potatoes were heated in a GC oven for 15 minutes at 160 and 180 ºC (Elballat *et al.*, 2020).

Storage condition also influences the formation of acrylamide, especially in potatoes. When potatoes are stored at low temperature, their L-asparagine content increases, which can leads to acrylamide formation, but to reduce the L-asparaginase content in potatoes, it should be stored at temperature higher than 10^{o}C. Increase in formation of acrylamide can also be due to range of storage temperature. When the temperature is low, there is increase in reducing sugars of some foods, like potatoes, thereby aids the formation of acrylamide. Therefore foods of such should be stored at temperature above 10^{o}C, so as to reduce the possible means of additions of reducing sugars (Elballat *et al.*, 2020).

Some chemicals are used to reduce acrylamide formation, decrease in acrylamide content in potatoes when sodium ascorbate and ascorbyl palmitate was demonstrated, it was reported to be due to reduced pH and increased binding water (Elballat, *et al.*, 2020). Addition of hydrochloric acid and citric acid reduced the ffomation of acrylamide, also showed that, the degradation of acrylamide that have been formed was increased (Elballat, *et al.*, 2020).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection and Pretreatment

Soil samples were collected from rhizosphere of matured plants (*Azadirachta indica, Moringa oleacea, Alstona boonei and Khaya senegalensis*), at the depth of 15 cm by ethanol and flamed sterilized cutlass, within the Botanical garden, University of Ibadan, Nigeria. The sampling points were located in the coordinates 7.457481 and 3.895045 for *Azadirachta indica*, 7.457481 and 3.895054 for *Alstona boonei*, 7.457970 and 3.895082 for *Moringa oleacea* and 7.458034 and 3.894965 for *Khaya senegalensis* (The map of each location is given in appendix 1- 4). The samples were collected in sterile polythene bags and brought to the laboratory for further analysis. Physical pre-treatment like, air drying, was done to encourage the growth of actinomycetes and reduce other bacterial load. This was done by measuring 10 g of each sample in a sterile foil paper and exposing to air for 72 hours (Alzahrani *et al.*, 2020).

3.2 Media preparation

Starch Casein Agar (SCA) and Actinomycetes Isolation Agar (AIA) were used for isolation. They were composed of (SCA) NaCl: 0.2 g, $MgSO_4.7H_2O$: 0.05 g, KNO_3: 2.0 g, Casein: 0.3g, K_2HPO_4: 2.0g, $CaCO_3$: 0.02g, soluble starch: 10 g, $FeSO_4.7H_2O$: 0.01 g, agar: 15 g, distilled water: 1000 mL, pH: 7.0; (AIA): Sodium caseinate 2.0 g, L-Asparagine 0.1 g, Sodium propionate 4.0 g K_2HPO_4: 0.5 g, $MgSO_4$: 0.1 g, $FeSO_4$: 0.001 g Agar 15 g, pH 8. The media were measured and mixed in Erlenmeyer flasks, plugged with cotton wool, covered with aluminum foil and sterilised in the autoclave at 121^oC for 15 minutes. The medium was allowed to cool to about 45^oC before adding streptomycin (5µg/mL) to inhibit the fungal and bacteria growth (Alzahrani *et al.*, 2020).

3.3 Isolation of the Actinomycetes from soil samples

Ten grams of each sample were diluted into 90 mL of distilled water to make 6 fold (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) dilutions, 1 mL of each dilution was measured into a Petri dish separately and 15 mL sterile prepared and cooled starch casein agar was poured into the plates in triplicate. This was done for each sample collected; the plates were then inverted and incubated at 27^{o}C for 3 to 5 days.The developed colonies were then observed, counted and recorded as CFU/mL of soil samples (Dhanam and Kannan, 2015).

3.4. Maintenance of pure culture

Colonies with suspected Actinomycetes morphology such as dry, hard, grey, gold and white powdery texture, branching filaments with or without aerial mycelia on the plates were picked and streaked separately on the SCA plate to obtain pure cultures which were then incubated at 27^{o}C. After which, each isolate obtained was kept in the slant medium and stored at 4^{o}C for further studies (Sanjotha, 2017).

3.5. Screening for L-asparaginase

3. 5.1. Plate assay method

The ability of the selected colonies to produce L-asparaginase were determined on M9 medium (Na_2HPO_4; 6.0 g, K_2HPO_4; 0.9 g, NaCl; 0.5 g, L-asparagine; 10 g, 1M $MgSO_4.7H_2O$- 2 mL, 0.1 M solution of $CaCl_2.3H_2O$; 1m L, 20% glucose stock; 10 mL, 0.005% phenol red, pH 6.5) and Glycerol Asparagine Medium (Glycerol; 1%, L- asparagine; 1%, K_2HPO_4; 0.1%, phenol red; 0.05%, pH; 6.5). The media were prepared both with agar and in broth, sterilized at 121^{o}C and inoculated with selected cultures. The plates and tubes were incubated at 25oCerature for up to 14 days and daily observations were noted visually. Plates and tubes with change in colour of the medium from yellow to pink were selected as L-asparaginase-producing plates and tubes, and the isolates on them were selected as L-asparaginase producers (Jeyaraj *et al*., 2020).

3.5.2. Quantitative screening through submerged fermentation.

One hundred millilitre of glycerol-asparagine broth in 250 mL conical flask was inoculated with 1.5x 10^{8} CFU/mL of 72hrs old actinomycetes suspension and incubated at 27°C in a shaker incubator at 200 rpm for 72 hours. At the end of the

fermentation period, the medium was centrifuged at 10,000rpm for 15 minutes and cell-free supernatant was taken as the crude enzyme (Saxena *et al.*, 2015)

3.5.3. Determination of the L-asparaginase Activity

The activity of the produced L-asparaginase enzyme was assessed according to the method of Saxena *et al.*, (2015), In this assay, the rate of hydrolysis of L-asparaginase was determined by measuring the ammonia released during Nessler's reaction. A 0.1 mL of crude extract was added to 0.2 mL of 0.05M Tris-HCl buffer (pH 8.6) and 1.7 mL of 0.01 M of L-asparagine. The mixture was incubated for 10 min at 37^{o}C and the reaction was terminated by the addition of 0.5 mL of 1.5M Trichloroacetic acid. The mixture was centrifuged at 10,000 rpm for 5 minutes and 0.5 mL of the supernatant was added to 7 mL of distilled water and treated with 1 mL of Nessler's reagent. Colour reaction was allowed to develop for 10 mins and the absorbance was read at 480 nm with a UV spectrophotometer. The amount of ammonia liberated was determined. One international unit of L-asparaginase enzyme was defined as the amount of enzyme that liberated 1um of ammonia per minute under the condition of assay (Saxena *et al.*, 2015).

$$Enzyme\ activity\ (IU) = \frac{Amount\ of\ Ammonia\ Liberated}{Incubation\ time\ x\ mL\ of\ enzyme\ used}$$

3.6. Screening for L-glutaminase-producing ability of selected L-asparaginase producers

The selected L-asparaginase producers were screened for their inability to produce L-glutaminase activity on M9 medium. This was done by replacing the L-asparagine (substrate) with L-glutamine and incubated for 30 days. Colonies with positive L-glutaminase activity (i.e. change plate from yellow to pink) were termed L-glutaminase producers and were screened out. Those isolates with no/low L-glutaminase activity (in which plates changed from yellow to pink after 7 days) were selected as no/ low-glutaminase L-asparaginase producers.

3.7. Identification of the isolates

Identification of selected isolates was done following standard International Streptomyces Project (ISP) procedure (Salo and Novero, 2020) and the results were compared with provision in the relevant section of Bergey's Manual of Systematic Bacteriology volume 5.

3.7.1 Morphological identification

An initial identification of the colony based on visual features was done by observing the size, shape, texture, odour and colour of the isolates, the microscopic identification was done by Gram staining and spore staining, and the slide was observed under a light microscope (x100) (Olutiola *et al*., 2000).

3.7.1.1 Gram staining

A drop of sterile water was placed on a sterile clean and grease-free glass slide; one loopful of each bacterium colony was placed on it to make a thin smear. The slide was air dried and heat-fixed by spirit lamp. The smear was thereafter flooded with crystal violet for 30 seconds and then rinsed with distilled water. It was then covered with Gram iodine solution for 1minute and rinsed gently with water. Next, the slide was destained with 95% alcohol and rinsed immediately with water. Then it was counter-stained with safranin for 30 seconds and rinsed.The slide was air-dried and observed under the light microscope (x100). Gram positive bacteria were characterised by purple colourisation while Gram negative were pinkish in colour (Cheesbrough, 2006).

3.7.1.2 Bacterial spore staining.

This is to detect the presence of bacteria endospores within vegetative bacterial cells. A loopful smear of each colony was made on different slides; heat-fixed and flooded with 5% Malachite green solution. The slide was steamed for 5 minutes with continuous addition of malachite green solution; it was then washed slowly with water and counter stained with 2 drops of Safranin solutions for 20 seconds. The slides were rinsed and allowed to dry. They were then examined under oil immersion objective (X100) lens. Endospores stained green while vegetative cells stained pink (Cheesbrough, 2006).

3.7.2 Biochemical examination of selected isolates

Biochemical test such as; Gelatine liquefaction, Casein hydrolysis, Starch hydrolysis, Oxidase test, Catalase test, Methyl red - Voges Prousker, Indole production, Nitrate reduction, Motility, Hydrogen sulphide test, Citrate utilization, Carbon sources utilization, and Sodium chloride tolerance were carried out to assist in microbial identification (Fawole and Oso, 2004).

3.7.2.1 Catalase Test

This was done to detect the presence of catalase enzyme which catalyses the breaking down of hydrogen peroxide to water and oxygen ($2H_2O_2 \longrightarrow 2H_2O + O_2$). Sterile inoculating loop was used to pick few drops of freshly prepared 3% hydrogen peroxide and dropped on a sterile clean slide; a loopful fresh culture was picked and then mixed together. The observation of the formation of white bubbles (O_2) would indicate the presence of catalase enzyme while the absence of bubbles indicates a negative result (Olutiola *et al*., 2000).

3.7.2.2 Oxidase Test

A loop full of freshly grown culture was rubbed on the filter paper moistened with oxidase reagent (phenylenediamine) and a loopful colony was picked to make a smear on it. The colour of the smeared portion was observed after 20 to 30 seconds. Colour change of smeared portion to a deep blue colour indicated a positive oxidase reaction i.e. the organism was able to produce an oxidase, which oxidised phenylenediamine in the reagent to a deep blue colour. No colour change indicated a negative reaction (Olutiola *et al*., 2000).

3.7.2.3 Starch hydrolysis

This was done to determine the ability of the isolates to produce enzymes that degrade starch. A 2.5 g soluble starch was mixed with 7.0 g nutrient agar to make 250mL, prepared and sterilised at 121^{o}C. The medium was poured into sterile plates, allow to set and test isolates were streaked on it. The plates were incubated for 3 days at 27^{o}C. After incubation, the plates were flooded with Gram's iodine. Hydrolysed starch appeared as a clear zones due to alpha amylase activity while reddish brown zones around the colony indicates a partial hydrolysis of starch (to dextrans), unhydrolysed starch formed a blue colour with the iodine (Olutiola *et al*., 2000).

3.7.2.4. Citrate Utilisation test.

This was done to check if the organism can use citrate as a sole source of energy and carbon. In principle, organic acid such as citrate (in Krebs' cycle) used as a carbon and energy source, alkaline carbonates and bicarbonates are produced immediately. The colour change of the indicator is due to alkaline production of acetic acd by the test organism, as its grown on the medium. Simmon citrate agar

(2.4 g) was dissolved in 100 mL of distilled water, homogenised and about 10mL was dispensed in each tube which were sterilised and allowed to cool in a slanted position for 24 hrs. The test organisms were inoculated onto each tube and incubated for 3 days. A changed from green colour of the media to blue indicated utilisation of the citrate (Olutiola *et al*., 2000).

3.7.2.5. Motility test.

Half strength of Nutrient agar (1.4 g/100mL) was prepared, homogenised, dispensed in test tubes, sterilised and was allow cooling. A sterile needle was used to pick some of a 48 old culture and was stabbed onto nutrient agar in tubes. The tubes were incubated at 25oCerature for 3 days. Motile bacteria gave diffused growth extending downward from the surface while non-motile bacteria had growth confined to the stab line (Cheesbrough, 2006).

3.7.2.6 Production of indole

It was done to check the ability of the bacteria to produce trytophanase, which break down the amino acid tryptophan to by-products that include indole. Tryptone broth (1 g) was prepared in 100 mL of distilled water, sterilised using autoclave at 121^{o}C for 15 minutes and allowed to cool. The broth was inoculated with test organism and an uninoculated tube was kept as control. The tubes were incubated at 25oC for 48 hours, then 1 mL of Kovac reagent was added, shaken gently after intervals of 10 to 15minutes and allowed to stand for few minutes to permit the reagent to come to the top. Then tubes were observed for cherry red layer at the top layer which indicates positive result (Olutiola *et al*., 2000).

3.7.2.7 Methyl red test

This was used to check the ability of the bacteria to perform mixed acid fermentation. Organisms that perform mixed acid fermentation produce enough acid to overcome the buffering capacity of the broth, thereby leading to decrease in pH. 5 mL of glucose phosphate broth (1 g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 mL distilled water) was dispensed in clean test tubes and sterilised. The tubes were inoculated with test organism and incubated at 25oCerature for 48 hours. At the end of incubation, few drops of methyl red solution were added to each test tube and change in colour was observed. A red colour indicated a positive reaction and yellow colour indicated negative reaction (Olutiola *et al*., 2000).

3.7.2.8 Voges-proskaeur test

This was done to distinguished bacteria on the basis of their production of acetoin, a neutral end product. 5 mL of glucose phosphate broth (1 g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 mL distilled water) was dispensed in clean test tubes and sterilised. The tubes were inoculated with test organism and incubated at 25oC for 48hours. At the end of incubation, 6% naphhtol and 6% Sodium hydroxide were added to about 1 mL of the broth culture. A strong red colouration formed within 30 minutes indicated positive reaction (Olutiola *et al*., 2000).

3.7.2.9 Gelatine liquefaction

This test was carried out for the detection of proteolytic activity. 10% gelatine was added to 100 mL of nutrient broth, homogenised and dispensed into test tubes, then sterilised at 121^{o}C for 10 minutes and allowed to cool. The broth was stabbed with the test organisms after cooling and was incubated for 3 days at 27^{o}C. After incubation, the tubes were refrigerated for 30 minutes to solidified any gelatine, after that observation for liquefaction was made and the tubes containing the fluidly gelatine was noted to be positive, while the tubes with solidified medium were noted to be negative. Gelatine media was prepared and sterilised using autoclave at 121°C (Olutiola *et al*., 2000).

3.7.2.10 Urease test

This was used to test the ability of the isolates to hydrolyse urea into carbon dioxide and ammonia. A 20 g of urease medium was dissolved in 100 mL of distilled water, homogenised, dispensed in bottles and sterilised at 121^{o}C for 15 minutes. After sterilisation, the bottles were allowed to set in a slanting position, freshly prepared cultures were picked, inoculated on each bottles and incubated for 2 days. A changed in colour from yellow to pink or red indicated a positive result (Olutiola *et al*., 2000).

3.7.2.11. Hydrolysis of casein

The protease activity was determined in a medium containing 1 g casein, 1 g glucose, 0.15 g K_2HPO_4 and 1.5 g agar in 100 mL of water (Sasikumar *et al.*, 2013). The isolates were stabbed on the medium and incubated at 28^{o}C for 3 days; proteolytic activity was identified by a clear zone around the colony (Adeoye *et al*., 2021).

3.7.2.12 Nitrate reduction test

This was used to test for the presence of the enzyme nitrate reductase, which causes reduction of nitrate in the presence of a suitable electron donor. Nitrate medium, consisting of peptone water and 0.1% potassium nitrate, was prepared. 5 mL of this medium was dispensed into test tubes with each tubes containing inverted Durham tubes and sterilised at 121^{o}C for 15 minutes and was allow to cooling, freshly grown pure culture was inoculated into each test tubes, the un-inoculated tube served as the control. The tubes were incubated at 25^{o}C for 3 days. After this, 2 drops of 1% sulphanilic acid in 5m acetic acid was added to each tubes followed by 2 drops of 0.6% dimethyl napthlamine in 5m acetic acid was added to the tubes. The development of a red colour shows the presence of nitrate and a positive result (Fawole and Oso, 2004).

3.7.2.13 Sugar Fermentation Test

This test was carried out to test for the various sugars utilising activity of the isolates. Each medium contained 1% of peptone water and 1% of appropriate sugar with phenol red as indication. The medium was homogenised, dispensed into clean test tubes and inverted Durham tubes were dropped into the each tube. The medium was sterilised at 121^{o}C for 10 minutes, and then allowed to cool, each test tube were inoculated with a fresh culture of each organism with un-inoculated tubes which served as the control. Acid production is shown by a change in the color of the indicator i.e. phenol red changes to yellow. If gas is produced, it accumulates in the Durham tubes (Olutiola *et al*., 2000).

The sugars used were Glucose, Galactose, Sucrose, Maltose, Lactose, Inositol, Fructose, Manintol, Arabinose and Raffinose.

3.7.3 Molecular identification

Molecular identification of the isolates was done using 16S rDNA gene sequence. (Tamura *et al*., 2007)

3.7.3.1 DNA Isolation.

The isolates were cultured in Starch casein agar medium broth for 72 hours and centrifuged at 10,000 rpm for 10 minutes, the filtrates were discarded and 0.5 mL

of lyzozymes was added into the tubes and mixed with vortexing machine for 5 minutes. The mixture was incubated for 5 minutes and shake again for 2 minutes, then 50 µl of proteinase K and 1.5 mL of CTAB buffer (composed of Ctab; 6.0 g, $NacL_2$; 24.54 g, tris; 3.63 g, EDTA; 1.75 g, distilled water; 300 mL, pH 8.0) was added. The mixture was incubated in a water bath at 65^{o}C for 60 minutes, 1.4 mL of chloroform.Isoamyalcohol (phenol) in the ratio 24:1 was added and then centrifuged at 10,000 rpm for 10 minutes. The upper layer was decanted with micropipette to prevent the DNA from mixing with it. 1.2 mL of Isopropanol was also added to the tubes to precipitate the DNA. The precipitated DNA formed a clot of strands in the solution. The solution was centrifuged at 10,000 rpm for 15 minutes and the supernatant was gently decanted. The DNA was washed with 0.9 mL of molecular grade 70% ethanol and vortex for 1 minutes and the mixture was centrifuged at 10,000 rpm for 10minutes. The ethanol was gently decanted and the tube with DNA was gently inverted to dry the ethanol. After drying the DNA was resuspended in 100 µL TE buffer (1 mL of Tris-Hcl, 200 µL of 0.5M EDTA and add up deionized water to make 100 mL) and left overnight in the fridge for further study (Tamura *et al*., 2007)

3.7.3.2 Gel electrophoresis

This was done to check if the DNA has been extracted. 1.5% agarose powder was mixed with 150 mL 1x TAE buffer, the mixture was heat in an oven, and 0.8 µL ethidium bromide was added to give colour. The mixture was allowed to cool and then poured gently inside the gel tray containing properly set comb, the gel was allow to polymerized for 30 minutes after which the comb was gently removed. 1X TAE buffer was poured into the gel tank to the point that covers the gel tray and the gel tray was carefully placed inside. 1 µL of DNA loading dye was added into each 4 µL of DNA sample and were loaded inside the well. The electrophoretic tank was closed and connected to electric field (60 V, 200 mA) for 1 hour. After 1 hour, the gel was gently removed and placed on UV detector to view the band (Tamura *et al*., 2007)

3.8. Physiological studies

The physiological factors (environmental and nutritional) influencing the L-asparaginase production were assessed and the optimisations were carried out using M9 medium broth as a Basal medium (Uzma *et al.*, 2016).

3.8.1. Effect of media

The effect of different production media (Tryptone Glucose yeast broth, Glucose Asparagine broth, M9 medium, Starch Casein Asparagine broth supplemented with Asparagine and MC-DOX medium) on growth and L-asparaginase production by the isolates were assessed. In this study, each medium was used as basal medium, 1.5 x 10^8 of 72 hours seed culture was inoculated into the medium, and the media were incubated at 25^{o}C on a shaker for 3 days. At the end of fermentation period, the growth of the isolates was determined spectrophotometrically at 600 nm and the enzyme was harvested by centrifugation at 10,000 rpm for 15 minutes. Enzyme activity was determined by Nesslerisation (Uzma *et al.,* 2016).

3.8.2. Effect of Temperature

The optimal temperature for the growth and production of L-asparaginase enzyme by selected isolates was studied by incubating inoculated basal media at 25, 30, 35, 40, 45 and 50°C separately. 1.5 x 10^8 of 72 hours seed culture was inoculated into the medium, the medium was incubated at different temperatures on a shaker for 3 days. At the end of the fermentation period, the growth of the isolates was determined spectrophotometrically at 600 nm and the enzyme was harvested by centrifugation at 10,000 rpm for 15 minutes. Enzyme activity was determined by Nesslerisation (Uzma *et al.,* 2016).

3.8.3. Effect of pH

The effect of pH on the growth and L-asparaginase production were studied by growing the selected isolates in basal medium of different pH (4, 5, 6, 7, 8, 9 and 10). Buffers used were Citrate buffer; pH 4 - 5, Phosphate buffer; pH 6-7, Tris-HCl buffer; pH 8, Carbonate buffer; pH 9 – 10. 1.5 x 10^8 of 72 hours seed culture was inoculated into the medium with different pH, the medium was incubated on a shaker at 25^{o}C for 3 days. At the end of the fermentation period, the growth of the isolates was determined spectrophotometrically at 600 nm. The enzyme was harvested by centrifugation at 10,000 rpm for 15 minutes. Enzyme activity was determined by Nesslerisation (Uzma *et al.,* 2016).

3.8.4. Effects of Incubation period

Effects of incubation period on the growth and L-asparaginase production by the selected isolates were studied on M9 medium after incubation at different time

intervals (Day 3, 5, 7, 10, 12, and 14). 72 hours seed culture (1.5 x 10^8) was inoculated into the medium, the medium was incubated at 25^{o}C on a shaker. The growth and enzyme activity was studied at different days. At the end of each period of fermentation, the growth of the isolates was determined spectrophotometrically at 600 nm and the enzyme was harvested by centrifugation at 10,000 rpm for 15 minutes. Enzyme activity was determined by Nesslerisation (Uzma *et al.,* 2016).

3.8.5. Effect of Carbon sources.

The influences of different carbon sources on efficient L-asparaginase production were assessed. Carbon sources like glucose, sucrose, maltose, lactose and starch, arabinose, mannitol were used. Each carbon source (0.2%) was added to the basal medium separately. A 1.5 x 10^8 of 72 hours seed culture was inoculated into the medium, the medium was incubated at 25^{o}C on a shaker for 3 days. At the end of fermentation period, the growth of the isolates was determined spectrophotometrically at 600nm and the enzyme was harvested by centrifugation at 10,000 rpm for 15 minutes. Enzyme activity was determined by Nesslerisation (Uzma *et al.,* 2016).

3.8.6. Effect of Nitrogen sources

The effect of different nitrogen sources (Peptone, Yeast extract, Potassium nitrate, Ammonium nitrate and Sodium nitrate) on asparaginase production was assessed. Each nitrogen source (0.2%) was added to the basal medium separately. A 1.5 x 10^8 of 72 hours seed culture was inoculated into the medium, the medium was incubated at 25^{o}C on a shaker for 3 days. At the end of fermentation period, the growth of the isolates were determined spectrophotometrically at 600nm and the enzyme was harvested by centrifugation at 10,000 rpm for 15 minutes. Enzyme activity was determined by Nesslerisation (Uzma *et al.,* 2016).

3.8.7 Effect of Inoculum sizes

The effects of inoculum sizes on L-asparaginase production were studied by inoculating the basal medium with different concentrations of the selected isolate broth (1.5 x 10^8 -1.2 x 10^9). The medium was incubated at 25^{o}C on a shaker for 3days. At the end of fermentation period, the growth of the isolates was determined spectrophotometrically at 600nm and the enzyme was harvested by centrifugation

at 10,000rpm for 15 minutes. Enzyme activity was determined by Nesslerisation (Uzma *et al.,* 2016).

3.8.8 Effect of agitation

The effect of speed of agitation on growth and L-asparaginase production by selected isolates were studied by incubating the inoculated basal medium at different agitation rate (100, 150, 200, 250, 300 rpm). A 1.5 x 10^8 of 72 hours seed culture was inoculated into the 100 mL of M9 mediumin a 250 mL Erlenmeyer flask; the media were incubated on a shaker at different revolution for 3 days. At the end of fermentation period, the growth of the isolates was determined spectrophotometrically at 600nm and the enzyme was harvested by centrifugation at 10,000rpm for 15 minutes. Enzyme activity was determined by Nesslerisation (Uzma *et al.,* 2016).

3.8.9 Effect of Substrate concentration.

The effects of different substrate concentration on the growth and L-asparaginase production by selected isolates were studied at L-asparagine of 0.25%, 0.50%, 1.0%, 1.5% and 2.0%. The basal medium was prepared by substituting the amount of the substrate (L-asparagine) at different concentration in it. At the end of fermentation period, the growth of the isolates was determined spectrophotometrically at 600nm and the enzyme was harvested by centrifugation at 10,000rpm for 15 minutes. Enzyme activity was determined by Nesslerisation (Uzma *et al.,* 2016).

3.8.10 Influence of metal ions

The Effect of different metal ions on the growth and L-asparaginase production were assessed. Chloride form of Ba^{2+}, K^{2+}, Mn^{2+}, NH^{2+}, Mg^{+}, Fe^{2+} and $ZnSO_4$ were used. 0.2% of each of these metals were added to the basal medium separately and used for fermentation. At the end of fermentation period, the growth of the isolates was determined spectrophotometrically at 600nm and the enzyme was harvested by centrifugation at 10,000rpm for 15 minutes. Enzyme activity was determined by Nesslerisation (Uzma *et al.,* 2016).

3.9 Production of L-asparginase

The L-asparaginasse was produced with best combination based on the result of interaction of different factors on enzyme activity. it was composed of Na_2HPO_4;0.6%, K_2HPO_4; 0.3%, NacL; 0.05%, L-asparagine 1%, yeast extract 1.5%, 1M $MgSO_4$; 0.2%, 0.1M $CaCl_2.H_2O$; 0.1%, 20% glucose stock; 1%.

3.10 Purification of L-asparaginase.

The crude enzyme was purified following three steps, salting out using ammonium sulphate, desalting by dialysis and gel filtration chromatography using Sephadex G- 50 (Ravi Verma *et al*., 2016). A 0-40%, 40-80%, 80-100% concentration (12.28 g, 14.18 g and 7.68 g respectively) of ammonium sulphate was dispensed into 50 mL crude enzyme in 250 mL Erlenmeyer flask and gently magnetically stirred until it dissolved. These flasks were kept at 4^oC overnight and then centrifuged at 10,000 rpm for 15 minutes at 4^oC. The supernatant was decanted and the precipitate was dissolved in 0.5M Tris- Hcl (pH 8.6) (Sahar *et al*., 2018).

The dissolved precipitate from each range of salt concentration was dialysed. At this stage, Dialysis bag was cut (5 cm long) and soaked in Tris- HCl buffer pH 8.6 overnight, 10 mL of dissolved precipitate was put in the bag, sealed tightly and soaked in 50 mL of the same buffer at 4^oC. The buffer was changed at 2 hours interval and was later left overnight at 4^oC. The enzyme activity and protein contents were determined. The dialyzed enzyme was further purified by applying it on Sephadex G-50 at 25^oC. 10 g of Sephadex powder was soaked in 100 mL of Tris- Hcl buffer pH 8.6 for 48 hours and part of it was then loaded on the column (15cm X 2cm). The column was equilibrated with the same buffer and the dialyzsd enzyme was loaded on. It was eluted with the same buffer at a flow rate of 0.5 mL/min. Fractions of 3 mL was collected and the protein and enzyme activity of each fraction was determined using Lowry assay at 660 nm and Nesslerisation at 480 nm.The fractions with higher activity were pooled together, freeze-dried and used for further studies.

3.11 Characterisation of L- asparaginase.

3.11.1 Impact of temperature on stability and activity of crude and purified L-asparaginase

The method of Mohamed *et al*., 2015 was used to check the impact of temperature on activity and stability of L-asparaginase. 0.1 mL of the enzyme was dispensed in

0.2 mL of Tris- HCl buffer pH 8.6 and 1.7 mL of 0.05M L-asparagine substrate, temperature of incubation was varied between 10^{o}C and 75°C for 60 minutes. The activity and stability of the enzyme was checked at interval of 10 minutes for 60 minutes by Nesslerisation method (Noura *et al*., 2020).

3.11.2 Impact of pH on activity and stability of purified and crude L-asparaginase

Different pH was used to check the activity and stability of the enzyme. The assay procedure was done by varying the pH of the medium. The pH 4 to 10 was used, using different buffer (Citrate buffer; pH4- 5, Phosphate buffer; pH6-7, Tris-Hcl buffer; pH 8, Carbonate buffer; 9 – 10). The stability and activity of the enzyme was checked at interval of 10 minutes for 60 minutes by Nesslerisation method (Noura *et al*., 2020).

3.11.3 Influence of inducers and inhibitors on activity and stability of purified and crude L-asparaginase

This was done by the addition of 0.1 mL of 1% solution of inhibitor such as Ascorbic acid, Urea, Triton X-100, Sodium azide, Tween 80, EDTA, Sodium deocyl sulphate to the assay mixture and stability and activity of the enzyme was checked at interval of 10 minutes for 1 hour by Nesslerisation method (Noura *et al*., 2020).

3.11.4 Influence of different metal ion on activity and stability of crude and purified L-asparaginase

0.2 mL of 0.2 M of different metal in the chloride form (magnesium chloride, potassium chloride, calcium chloride, Iron Chloride, mercury chloride, sodium chloride, Zinc chloride, and Ammonium chloride was added to the asay procedure to determine the effects of metal ions on stability and activity of the enzyme.The activity was checked at interval of 10 minutes for 1hour by Nesslerisation method (Prakash *et al*., 2020).

3.11.5 Effect of Substrate concentration on activity and stability of crude and purified L-asparaginase

1.7 mL of various concentrations (4.0 mM, 8.0 mM, 1.2 mM, 1.6 mM and 2.0 mM) of L- asparagine was used for the assay mixture and their effect was checked on stability and activity of the enzyme at interval of 10 minutes for 1hour by Nesslerisation method (Muneer *et al.,* 2020).

3.11.6. Influence of amino acids on L-asparaginase

The Specificity of the enzyme towards the different substrate was determined by incubating the enzymes with 1.7 mL of 0.05 M of various amino acids like L-aspartic acid, L- arginine, L- phenylalanine, L- asparagines and L- glutamine and L-asparagine and the stability and activity as observed at interval of 10 minutes for 1 hour by Nesslerisation method (Muneer *et al.,* 2020).

3.11.7. Effect of enzyme concentration on activity and stability of crude and purified L-asparaginase

Different concentration (20%, 40%, 60%, 80%, and 100%) of enzyme was used in the assay mixture reaction to check their effect on the enzyme stability and activity and this was done by Nesslerization method at the interval of 10minutes for 60 minutes. 10 mL of the enzyme was dispensed in to graduated vials and reduced into different level to make different concentrations.

3.11.8. Molecular Weight Determination of L-asparaginase.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was used to evaluate the molecular weight of the crude enzyme and to determined the purity of the purified enzyme. 10% gel was prepared. The gel was stained with Coomassie Brilliant Blue R-250. The protein ladder (cat 26614) was used as reference marker. The material used were; sample buffer(Tris-HCI (pH 6.8) buffer 0.4 mL, 10% SDS 2.5%, 2-mercaptoethanol 0.4 mL, Glycerol 2.0 mL, Bromophenol blue 0.002 g, Distilled water 4.7 mL), Electrode buffer (Tris-HCI 6.05 g, SDS 2 g, Glycine 28.8 g, Distilled water 2.0 L), Separating (4x) gel buffer (Tris-HCl 18.3 g, distilled water 100 mL, pH 8.8), Stacking (4x) gel buffer (Tris-HCl 6.055 g, distilled water 100 mL, pH 6.8), (30%) Bisacrylamide (29.2 g acrylamide, 0.8g of bis-acrylamide, 100 mL).

The procedure is as follows; separating gel that comprises of (Distilled water 19.5 mL, Bisacrylamide (30%) 10 mL , 4x separating gel buffer 10 mL , SDS (10%) 0.8 mL, Glycerol (10%) 0.35 mL, TEMED 20 µL, APS (2%) 0.6 mL) were mixed and

gently poured in a vertical mould, the saturated butanol was added and the gel was allowed to polymerise. After 30 minutes, the butanol was removed and upper portion of gel was washed with deionized water. the stacking gel (Comprised of Distilled water 6.3 mL, Bisacrylamide (30%) 2 mL, 4x separating gel buffer 2.5 mL, SDS (10%) 0.2 mL, Glycerol (10%) 0.15 mL, TEMED 10 µL , APS (2%) 0.13 mL) was poured on the separating gel on the vertical mould and comb was placed in it, the gel was allowed to polymerised and the comb was removed. 30 µL of enzyme was mixed with 4X lamelli reducing buffer to give it colour, the sample was heated at 98^{o}C for 10 minutes and 15 µL of sample was loaded in the wells. Electrophoresis was carried out at 50V until when the dye front reached the separating gel and the voltage was increased to 100V. After the run is complete the gel was taken out and washed with water. Then comassive blue staining was carried out. The staining solution consisted of 90 mL water, 90 mL methanol, 10 mL acetic acid and 0.25 g Commassive blue dye. While the destaining solution consisted of 90 mL water, 90 mL methanol, and 10 mL acetic acid. The gel was placed in 100 mL of staining solution for 30 minutes (for staining the protein in the gel) and removed; it was then placed in the destaining solution for destaining the gel overnight (El-Fakharany *et al*., 2020).

3.11.9. HPLC Analysis of the partially purified enzyme

HPLC analysis of crude and purified L-asparaginase was performed on an Agilent series 1100 HPLC system fitted with a reversed phase high performance liquid chromatography (RP-HPLC) column, 3.9 mm X 75 mm length filled with Atlantis C18, 5 µm, 300A (Agilent). The solvent system was prepared as solvent A containing 0.1% (v/v) formic acid in H_2O and solvent B containing 0.1% (v/v) acetonitrile (ACN). It was eluted by gradient elution of solvent B in 6 min as 70% at 0 to 1 min, 70 to 95% at 5 to 8 min, 95% at 8 min to 10 min, 95 to 70% at 10 to 15 min at flow rate 1.0 mL per min.

3.11.10. Fourier Transformed Infra-red (FTIR) Spectroscopy.

The surface properties and functional groups of the enzyme was determined by using Fourier Transformed Infra-red (FTIR) Spectroscopy (Agrawal and Kango, 2019)

3.12. Biological activities of L-asparaginase.

Different biological activity was carried out on both the crude and purified enzyme.

3.12.1 Antibacterial potential of L-asparaginase

This was done to check the effect of the enzyme on different pathogenic bacteria. 200µL of the enzyme was dispensed into a sterile 96well plate; 100 µL of Muller Hilton Broth (MHB) was also added to the remaining well, 100 µL of the enzyme in the first well was diluted with MHB broth in the other well to make different concentration. 10 µL of each test organism (*Salmonella typhi* ATCC14028, *Escherichia coli* ATCC 25922, *Pseudomonas aeroginosa* ATCC 10145, *Bacillus subtilis* ATCC 23857, *Enterococcus faecalis, Staphylococcus aureus* ATCC 6571 that was collected from Microbiology Laboratory, International Centre for Chemical and Biological Sciences, University of Karachi, Pakistan) was diluted with 100 mL of MHB and 100 µL was dispensed into each well. The plates were incubated for 17 - 24 hours at 37^{o}C after which 20 µL of alamar blue dye was added to it. The plate was further incubated for two hours on a shaker, and then the activity was checked with a spectrophotometer at 570 nm and 600 nm.The wells with viable cells were change from blue to pink and the wells with dead cells retained the blue colour of the dye.

3.12.2. Antifungal activity of L-asparaginase

Sabroud dextrose agar (32.5 g) was dispensed in 500mL of distilled water. The medium was homogenized and 4mL of it was dispensed in screw capped test tubes. The tubes were sterilised by autoclaved at 121^{o}C for 15 minutes and was allowed to cool up to 35^{o}C. The enzyme (200 µL) was carefully dispensed into the tubes and placed in slanted position for 24 hrs. Each tube was then inoculated with the test fungus and incubated between 27^{o}C and 29^{o}C for 7 days. Tubes without enzyme served as control. Fungi used were; *Candida albican* ATCC 36082, *Microsporum canis* ATCC 10214, *Trichophyton rubrum* ATCC 10214, *Fusarium lini* ATCC NRRL 2204, *Aspergillus niger* ATCC 1015 (These organisms were collected from Bioactivity Laboratory, International Centre for Chemical and Biological Sciences, University of Karachi, Pakistan). The growth of the organisms were then observed in the tubes and measured using linear growth measurement.

3.12.3. Insecticidal activity of the L-asparaginase

Crude enzyme sample (200 µL) was poured in the filter paper in the petri dish, the filter paper was allowed to dry for 24 hours at 25^{o}C and 10 pieces of each rice

infesting insect in storage (Adult stage of *Sytophillus* and *Ryzopertha* species collected from Bioactivity Laboratory, International Centre for Chemical and Biological Sciences, University of Karachi, Pakistan) was added into the petri dish. The dish was sealed with parafilm and viability of the insect was observed after 24 hours (Ligia *et al*.2015). The plate with filter paper treated with distilled water served as the control.

$$\%\text{Mortality} = 100 - \frac{\text{Alive insects}}{\text{Total}} \times 100$$

3.12.4. Brine shrimps lethality assay.

This was used to check the cytotoxic effect of the enzyme. Sea water (34.7 g of sea salt, distilled water, pH 7.4) was poured in a hatching tray; 5 g of shrimp's egg was poured in it and was placed in the hatching chamber (fitted with lamp to attract brine shrimp larvae) for 48 hours between 25^{o}C and 30^{o}C. After 48 hours, different concentrations of the enzyme were made in vial in triplicate and 10 larva were placed in each bottle and the bottles were supplemented with sea water. This was incubated at 25^{o}C for 24 hours and the viability of the larva was observed (Osama *et al*., 2020)

3.12.5. Antileishmanial potential of L-asparaginase

The pure culture of Leishmania (collected from Bioactivity Laboratory, International centre for Chemical and Biological Sciences, University of Karachi, Pakistan) was taken into the 15 mL of rpmi-1640 medium and it was centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded and freshly prepared rpmi-1640 was added to the pellet. RPMI medium (180 µL) was dispensed into the first well of the 96 well plate and 100 µL in other well. The enzyme sample (20 µL) was added to the first well and dilution was made to other wells from it.100 µL of the cell suspension was added to the wells and plate was incubated for 72 hours. The well without the enzyme served as control. After incubation, 10 µL of MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT dye) was added to all the wells and the plate was incubated at 18^{o}C -37^{o}C for 4 hours. After incubation, all the media in the well was removed and 100 µLof dimethyl sulfoxide (DMSO) was added and then incubated again for 10 minutes. The colour change of the dye from blue to yellow was observed and the activity was read

spectrophometrically at 540 nm and the percentage of inhibition was calculated (Singh *et al*., 2017).

3.12.6. Antioxidant activity of L-asparaginase

This was done to check if the hydrogen atom in the enzyme has the ability to reduce the electron in nitrogen atom of the radical. In this assay, 1, 1, Diphenyl-2picrylhydrazyl (DPPH) was used as radical. DPPH +AH $\longrightarrow$ DPPH – H +A

The enzyme (10 µL) was dispensed in 96 well plates and the absorbance value was read at 517 nm in the spectrophotometer. DPPH solution (90 µL) was then dispensed in the wells, incubated at 37ºC for 30 mins, the plate was again read at 517 nm and the inhibitory percentage was calculated. The well with no enzyme was used as control while Gallic acid and N-acetyl L-cystein served as standard. DPPH appeared blue when prepared in ethanol but when it comes in contact with an antioxidant molecule, turned to yellow. All the experiment were done in triplicates (Nongkhlaw and Joshi, 2015)

Scavenging effect (%) = [(Ao–A1)/Ao] x100

3.12.7. *In vitro* anticancer activity of L-asparaginase from *Amycolatopsis japonica and Sphingobium yanokuiye.*

This was done to check the activity of the crude and partially purified enzyme on three cell lines; 3T3 cell line (Normal cell line), AUB5 cell line (breast cancer cell line) and $Caco_2$ cell line (Colon cancer cell line). Cell lines were collected from the cell culture bank, Panjwani Centre for Molecular Medicine and Drug Research, ICCBS, Karachi, and were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% Non-essential amino acid and incubated at 37ºC in 5 % CO_2 incubator. DMEM was replaced every 48-72 hour until 80% confluency was achieved. The cytotoxicity of samples against the three cell lines were determined by the MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. 1×10^5 cell/mL cell suspension (100 µL) were seeded in round bottom 96 well plate at the density of 10000 cell/well and incubated at 37 ºC in 5 % CO_2 incubator. After 48 hours of incubation, the cell has fully grown and attached to the wells. The medium was removed, various concentrations of crude and purified L-asparaginase (100, 50, and 25 µL) was mixed with the DMEM medium (100, 150, and 175 µL) respectively such that total volume of 200 µL

solution were dispensed in their respective wells and incubated for 24 h at 37°C in 5% CO_2 incubator. An untreated well served as positive growth control. After 24h incubation, all wells were viewed under fluorescence microscope (Nikon Eclipse TS100 Inverted microscope), images were taken and the whole solution from each well was removed. 2 mL of MTT dye (5 mg/mL) were 10 times diluted with fresh DMEM media such that this medium contain 0.5 mg/mL MTT dye and 200 µL of this solution were dispensed in each wells and further incubated for 2 h at 37°C in 5% CO_2 incubator. After the incubation, the medium containing MTT dye were removed and 100 µL of dimethyl sulfoxide (DMSO) was added to all wells to solubilise the formed formazan crystals and absorbance were recorded at 570 nm in spectrophotometer (Multiskan GO, Thermo Scientific). The cytotoxicity (%) of the enzyme was checked by relating with the untreated positive growth control. The plot of % cytotoxicity/inhibition versus sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (IC_{50}). All experiments were performed in triplicate (Jenila and Gnanadoss, 2020).

$$\% \text{ cytotoxicity/inhibition} = 100 - \frac{\text{O.D of treated well} - \text{O.D of media control}}{\text{O.D of untreated control} - \text{O.D of media control}} \text{ X } 100$$

3.12.8. Acrylamide reduction potential of L-asparaginase

Inhibition of acrylamide formation in fried food strips prepared from freshly peeled potatoes were oven-dried at 85ºC for 10 min, and then 100 g of the strips were soaked in 100 mL of the enzyme solution. As control, 100 g of dried potatoes were soaked in double distilled water. Subsequently enzyme-treated and untreated potato strips were cooked by frying in 100 mL of oil (175ºC) for 15 minutes. The fried strips were crushed and placed in 50mL of ethanol and mixed thoroughly to extract acrylamide. The ethanol extract was concentrated by vacuum evaporation and analyzed by gas chromatography (GC) for acrylamide using a Stable Wax DA column with FID detector and helium gas as carrier. The presence of acrylamide was determined by checking each prominent peak from GCMS library and the quantity present was calculated from the height of the peak (Jiao *et al*., 2021).

CHAPTER FOUR

RESULTS

4.1. Isolation of Bacterial from rhizospheric soil samples

A total of 145 microorganisms were isolated from the eight rhizospheric soil samples (Table 4.1). The samples designated as RSSAI (a and b), RSSAB (a and b), RSSKS (a and b) and RSSMO (a and b). From the rhizospheric soil of *Azadirachta indica* sample (RSSAIa), the highest CFU/mL of soil was (1.8×10^8). The sample had twenty morphologically distinct isolates, while the RSSAI (b) sample yielded (2.4×10^7) CFU/mL with ten morphologically distinct isolates. The rhizospheric soil of *Alstona boonei* sample (RSSABa) CFU/mL of soil was 1.2×10^7 with eight morphologically distinct isolates while the RSSAB (b) sample yielded (1.2×10^7) CFU/mL with eight morphologically distinct isolates. The CFU/mL of soil from the sample of rhizospheric soil from *Khaya senegalensis* (RSSKSa) was 1.3×10^8 with twenty morphologically distinct isolates, while that from (RSSKSb) sample gave (1.3×10^8) with seventeen morphologically distinct isolates. From the rhizospheric soil of *Moringa oleacea* sample (RSSMOb), the highest CFU/mL of soil was (1.5×10^8). The sample had fourty morphologically distinct isolates, while the RSSMO (a) sample yielded (1.3×10^8) CFU/mL with twenty morphologically distinct isolates.

Table 4.1: Bacteria count in soil samples obtained from medicinal plants rhizosphere at the Botanical garden, University of Ibadan.

Source/Sample code	Bacteria (Log of cells)	No. of isolates
RSSAI(a)	1.8×10^8	20
RSSAI(b)	2.4×10^7	10
RSSAB(a)	1.2×10^7	8
RSSAB(b)	3.6×10^7	10
RSSKS(a)	1.3×10^8	20
RSSKS(b)	1.3×10^8	17
RSSMO(a)	1.3×10^8	20
RSSMO(b)	1.5×10^8	40
	Total	145

RSSAI (a): Rhizospheric soil sample from *Azadirachta indica* point a

RSSAI (b): Rhizospheric soil sample from *Azadirachta indica* point b

RSSAB (a): Rhizospheric soil sample from *Alstona boonei* point a

RSSAB (b): Rhizospheric soil sample from *Alstona boonei* point b

RSSKS (a): Rhizospheric soil sample from *Khaya senegalensis* point a

RSSKS (b): Rhizospheric soil sample from *Khaya senegalensis* point b

RSSMO (a): Rhizospheric soil sample from *Moringa oleacea* point a

RSSMO (b): Rhizospheric soil sample from *Moringa oleacea* point b

4.2. Detection of L- asparaginase production by the isolates obtained from rhizospheric soils.

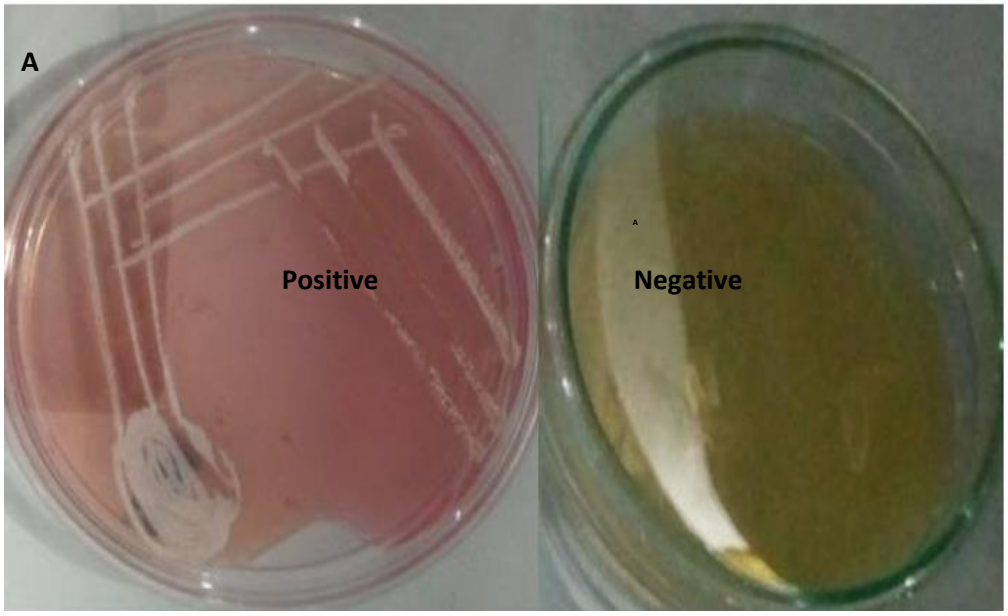
The abilities of the isolates obtained to utilise L-asparagine as a sole Nitrogen source is reported in Table 4.2. It was observed that, out of the 145 isolates obtained, 67 isolates that showed L-asparaginase producing ability on M9 medium and Glycerol L-asparaginase medium. This was noticed by colour change (initial yellow colour to pink) (Fig 4.1a and b). Out of the distinct colonies obtained from RSSMO, (46%) had positive L-asparaginase production in either Glycerol - Asparagine medium or M9 Medium, of which (42% and 17%) excellent production (+++) was observed on M9 medium and Glycerol-Asparagine medium respectively. 40% of the distinct colonies from RSSKS are L-asparaginase producers, out of which 60% showed excellent production on M9 medium. Out of the distinct colonies obtained from RSSAI, (47%) had positive L-asparaginase production in either Glycerol –Asparagine medium or M9 Medium, of which (50% and 14%) excellent production (+++) was observed on M9 medium and Glycerol-Asparagine medium respectively. 40% of the distinct colonies from RSSAB are L-asparaginase producers, out of which 50% showed excellent production on M9 medium and 20% showed excellent production on Glycerol-Asparagine medium (Table 4.2).

Positive producers were selected for further screening based on differential medium support at different days' interval on M9 medium (broth and Agar). Fifteen out of the isolates showed good consistency starting from 24 hours to 96 hours of incubation in that; there was change in colour of the medium both on agar and broth from yellow to pink at the same hour. Based on these, isolates RSSMO(b)5, RSSMO(a)4, RSSMO(b)4, RSSAB(b)3, RSSKS(b)1, RSSAB(b)2, RSSAI(a)2, RSSMO(B)8, RSSKS(b)8, RSSMO(b)37, RSSKS(a)12, RSSAI(b)3, RSSAB(a)2, RSSMO(B)2 and RSSKS(a)4 were picked as good producers of L-asparaginase as seen in Table 4.3.

The L-glutaminase activity of the selected isolates relative to incubation duration was determined as seen in Table 4.4. From the result, out of 15 selected isolates, 33.5% of isolates showed L-glutaminase activities between 5 to 10 days of incubation, while 66.5% showed no L-glutaminase activity over 10 days. It was also noticed that between 15 and 20 days up to 40-60% of the isolates showed L-

glutaminase activity. After 30 days of incubation, 80% of the isolates were observed to be L-glutaminase producers. Isolates RSSMO(a)4, RSSKS(b)1, and RSSMO(b)8 showed no glutaminase activity after 30 days of incubation, Isolate RSSMO(b)5, showed no L-glutaminase activity after 25 days of incubation, quantitatively gave the highest L-asparaginase yield (0.2772 ± 0.001U/mL) after 3 days of submerged fermentation. RSSMO(b)8, though completely negative to L-glutaminase activity over 30 days, quantitatively, the L-asparaginase yield by day 3 was quiet low (0.60 ± 0.001U/mL)

The result after 3 days of fermentation revealed that the yield of the L-asparaginase varied with the isolates. The yield ranged from the highest value of 0.2772 ± 0.001 U/mL to the lowest value of 0.0252 ± 0.001 U/mL. Isolates RSSMO(b)5 gave 0.2772 ± 0.001 U/mL followed by isolate RSSKS(a)1 and RSSKS(a)4 with the yield 0.231 ± 0.001 U/mL and 0.210 ± 0.01 U/mL respectively. The lowest yield was observed in isolate RSSKS(b)1 with the value of 0.0252 ± 0.001 U/mL followed by isolated RSSAI(b)3 and RSSAB(b)2 with the value of 0.042 ± 0.001 U/mL and 0.050 ± 0.0001 U/mL respectively (Table 4.4).



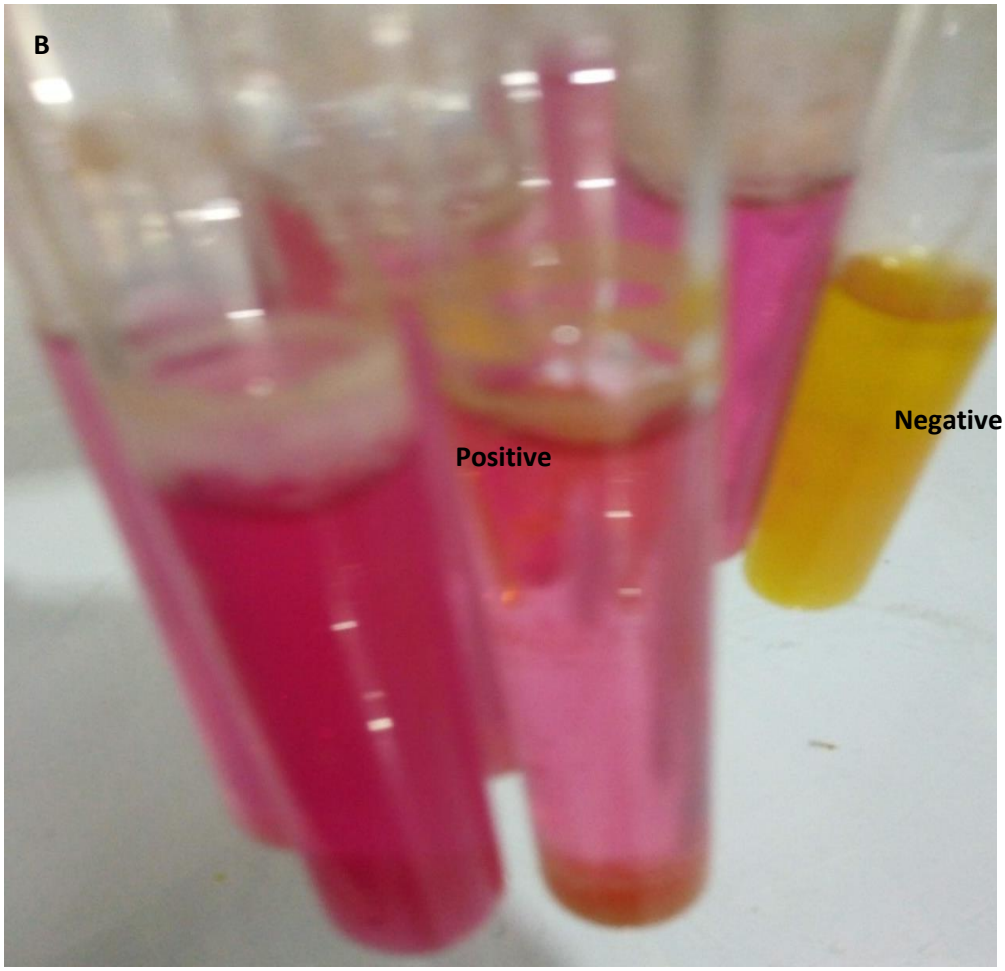


Plate 4.1A and B: Screening for L-asparaginase producing isolates on plate and tubes

Table 4.2: Screening of Bacteria from different medicinal plant Rhizospheric soil for L-asparaginase production.

Isolates code	Screening on Glycerol asparagine agar plate	Screening on Glycerol Asparagine broth	Screening on M9 medium agar plate	Screening on M9 medium broth
RSSMO(a)3	+	++	_	_
RSSMO(a)4	+	++	++	+++
RSSMO(a)7	+	++	_	_
RSSMO(a)11	+	_	++	++
RSSMO(a)13	+	++	_	_
RSSMO(a)13	+	+++	++	+++
RSSMO(a)14	_	++	++	+++
RSSMO(a)15	_	+	+	++
RSSMO(a)20	+	+++	++	+++
RSSMO(b)	+	++	++	+++
RSSMO(b)4	+	+	_	_
RSSMO(b)5	++	++	++	+++
RSSMO(b)6	+	_	+++	+++
RSSMO(b)7	+	+++	++	++
RSSMO(b))8	+	++	++	+++
RSSMO(b)9	_	+	++	++
RSSMO(b)11	_	++	++	+++
RSSMO(b)16	+	+	_	_
RSSMO(b)19	+	_	_	_
RSSMO(b)19	+	+	+	+
RSSMO(b)21	+	+	_	_
RSSMO(b)21	+	+	++	+++
RSSMO(b)22	+	+	++	_
RSSMO(b)25	+	+++	_	_
RSSMO(b)28	+	++	_	_
RSSMO(b)30	_	_	+	+
RSSMO(b)33	+	++	++	+++
RSSMO(b)37	+	+++	++	+++
RSSKS(a)1	+	++	++	_
RSSKS(a)4	+	++	+++	+++
RSSKS(a)5	+	_	_	_
RSSKS(a)6	+	_	++	+++
RSSKS(a)12	+	++	++	+++
RSSKS(a)15	+	++	_	_
RSSKS(a)19	+	+	_	_
RSSKS(a)20	+	_	_	_
RSSKS(b)1	+	++	++	+++
RSSKS(b)3	+	+	+++	+++
RSSKS(b)6	+	_	_	_
RSSKS(b)8	+	++	++	+++
RSSKS(b)9	+	+	+++	+++
RSSKS(b)I0	+	_	++	+++
RSSKS(b)14	+	+	++	+++

RSSAI(a)1	_	_	+	+
RSSAI(a)2	+	++	+++	+++
RSSAI(a)3	+	_	+++	+++
RSSAI(a)5	+	_	+++	+++
RSSAI(a)16	+	+++	_	_
RSSAI(a)17	+	+++	_	_
RSSAI(a)18	+	+	++	+++
RSSAI(a)19	+	+	++	+++
RSSAI(a)20	_	+	++	+
RSSAI(b)2	+	_	++	_
RSSAI(b)3	+	++	++	+++
RSSAI(b)8	+	++	+++	+++
RSSAI(b)9	+	+++	_	_
RSSAI(b)10	+	++	++	_
RSSAB(a)2	+	+	++	+++
RSSAB(a)4	+	_	++	++
RSSAB(a)5	+	+	+	+++
RSSAB(a)7	+	+++	_	_
RSSAB(a)8	+	_	-	_
RSSAB(b)1	+	_	+++	+++
RSSAB(b)2	+	+	+++	+++
RSSAB(b)3	+	+	+++	+++
RSSAB(b)4	+	+++	_	_
RSSAB(b)7	+	++	++	++

Key:

+	Good producers
++	Very Good producers
+++	Excellent producers

Table 4.3: Screening on M9 medium of Bacteria selected based on differential media support for L-asparaginase production.

Isolates code	M9 Agar				M9 Broth			
	24 (hrs)	48 (hrs)	72 (hrs)	96 (hrs)	24 (hrs)	48 (hrs)	72 (hrs)	96 (hrs)
RSSMO(B)4	+	+	+	+	+	+	+	+
RSSKS(b)9	+	+	+	+	_	_	+	+
RSSMO(B)5	+	+	+	+	+	+	+	+
RSSAB(b)3	+	+	+	+	_	+	+	+
RSSAI(a)20	_	_	+	+	_	_	+	+
RSSAI(a)18	+	+	+	+	_	_	_	+
RSSKS(b)14	+	+	+	+	_	_	+	+
RSSMO(a)4	+	+	+	+	+	+	+	+
RSSAB(a)2	+	+	+	+	+	+	+	+
RSSAB(b)2	+	+	+	+	+	+	+	+
RSSAI(b)3	+	+	+	+	+	+	+	+
RSSAI(a)2	+	+	+	+	+	+	+	+
RSSMO(B)8	+	+	+	+	_	_	+	+
RSSKS(b)8	+	+	+	+	+	+	+	+
RSSMO(B)33	+	+	_	+	_	_	+	+
RSSMO(B)37	+	+	+	+	_	+	+	+
RSSMO(B)21	+	+	+	+	_	+	+	+
RSSMO(a)20	_	+	+	+	_	_	_	+
RSSKS(b)1	+	+	+	+	+	+	+	+
RSSMO(a)13	_	+	+	+	_	_	+	+
RSSKS(a)4	+	+	+	+	+	+	+	+
RSSKS(a)12	+	+	+	+	_	+	+	+
RSSKS(b)3	_	-	+	+	_	_	_	+

Key:

\+ Positive
\- Negative

Table 4.4: Screening of selected Bacteria for L-glutaminase activity relative to time and secondary screening of L-asparaginase by submerged fermentation.

Isolate code	Time(days)/ L- glutaminase activity						Submerged fermentation (U/mL)
	5	10	15	20	25	30	3 days
RSSMO(B)5	-	-	-	-	-	+	0.277 ± 0.001
RSSMO(a)4	-	-	-	-	-	_	0.134 ± 0.001
RSSMO(B)4	-	-	_	+	+	+	0.059 ± 0.001
RSSAB(b)3	+	+	+	+	+	+	0.042 ± 0.001
RSSKS(b)1	_	_	_	_	_	_	0.134 ± 0.001
RSSAB(a)2	+	+	+	+	+	+	0.109 ± 0.001
RSSKS(a)4	_	_	+	+	+	+	0.210 ± 0.01
RSSAB(b)2	_	_	_	+	+	+	0.237 ± 0.0001
RSSAI(a)2	+	+	+	+	+	+	0.067 ± 0.0001
RSSMO(B)8	-	-	-	-	-	-	0.060 ± 0.0001
RSSKS(b)8	_	_	_	+	+	+	0.025 ± 0.0001
RSSMO(B)37	+	+	+	+	+	+	0.085 ± 0.0001
RSSMO(B)21	_	_	_	+	+	+	0.097 ± 0.0001
RSSKS(a)12	+	+	+	+	+	+	0.050 ± 0.0001
RSSAI(b)3	-	-	-	-	+	+	0.151 ± 0.001

Key: *Each value is a Mean of Triplicate production standard Error

\+ Positive - Negative

4.3. Identification of the isolates

Based on the above results six isolates with no or low glutaminase activity were selected as L-asparaginase-producers and used for further analysis. They were RSSMO(b)5, RSSKS(a)4, RSSKS(b)1, RSSAI(b)3, RSSAB(b)2, RSSMO(a)4. Isolate RSSMO(b)5, which was found to be the best producers of L-asparaginase of all the isolates that were screened was a white small size colony with round shape (Table 4.5). The colony was dried, leathery and gave earthy odour. At maturity, it gave a pink pigment. RSSMO(a)4 was observed to be small dried colony with gold colour, the colony stick to the medium and turn to brown upon maturation on starch casein agar. The colony gave ginsomin smell. Isolate RSSKS(b)1 was observed to be a big, wet, round, raise colony with yellow color, but produced pigment. Isolate RSSAI(b)3 had brownish colonies which were observed to be small, dried and produce golden pigment. Isolate RSSAB(b)2 was observed to be a small dull, white colony. The colony was raised; wet, shiny but showed no pigment. Isolate RSSKS(a)4 had golden colonies, the colonies appeared to be raised, big, dry and produced yellow pigment.

Table 4.5: Morphological characetrisation of selected L-asparaginase producers from rhizospheric soils.

Isolates codes	Morphological Characteristics of the selected isolates
RSSMO(B)5	White, dried, round, leathering pinpoint colony with earthy odour and branched filaments that extended in form of trees.
RSSAI(b)3	Small, brown, dried colony with golden pigment
RSSMO(a)4	Gold small colony that stick to the medium with ginsomin smell
RSSKS(a)4	Gold, raised, big, dry colony that grows into the medium. The produce yellow pigment
RSSKS(b)1	Big, round, wet, raised, yellow colony
RSSAB(b)2	Dull white, small, round, raised, wet colony

Keys

RSSAI (a): Rhizospheric soil sample from *Azadirachta indica* point a

RSSAI (b): Rhizospheric soil sample from *Azadirachta indica* point b

RSSAB (a): Rhizospheric soil sample from *Alstona boonei* point a

RSSAB (b): Rhizospheric soil sample from *Alstona boonei* point b

RSSKS (a): Rhizospheric soil sample from *Khaya senegalensis* point a

RSSKS (b): Rhizospheric soil sample from *Khaya senegalensis* point b

RSSMO (a): Rhizospheric soil sample from *Moringa oleacea* point a

RSSMO (b): Rhizospheric soil sample from *Moringa oleacea* point b

Biochemically, isolate RSSMO(b)5 was Gram positive, which could not produce acid from sugars (L-galactose, maltose, mannitol, lactose and glucose) except fructose, but hydrolysed starch, utilised nitrate but was unable to degrade gelatin (Table 4.6). It showed high rate of motility when stabbed on Nutrient agar and was identified as an Actinomycetes, which belong to *Pseudonocardioses* family and the genus *Amycolatopsis* after molecular studies and blast search through the gene bank of National Centre for Biotechnology Information (NCBI). Phylogenetic analysis of the isolate using maximum likelihood comparism with other genus of *Amycolatopsis* deposited in the gene bank revealed 90% similarity chart with *Amycolatopsis japonica* (Table 4.7).

The morphological and biochemical studies of the isolate RSSMO(a)4 revealed it was Gram positive rod with small short filament that joined together to form short trees. It showed resistant to degradation of cells by potassium hydroxide and unable to hydrolysed urea and could not withstand salinity as it was not able to withstand condition greater than 10%, and it was also observed that the isolate did not grow in strong acidic condition as no growth was observed when in a culture medium less than pH6. RSSMO(a)4 utilized some sugars like maltose, mannitol, lactose, glucose, fructose, raffinose and inositol but did not ferment galactose, sucrose and arabinose. It was motile and able to hydrolyse starch and gelatine but did not degrade nitrate (Table 4.6). The molecular identification of isolate using the genetic sequence of the 16sRNA primer confirmed the isolate to be *Stenotrophomonas pavani* after blast search through the gene bank of NCBI and the phylogenetic analysis of the sequence using maximum likelihood comparism revealed 94.55% similarity of the isolate to *Stenotrophomonas pavani* (Table 4.7).

Isolate RSSKS(b)1colony appeared to be gram negative cocci when viewed under a microscope, although no spore were seen. The biochemical characterization of the isolates revealed that it could utilised galactose, maltose, lactose, glucose and fructose but did not utilise mannitol, sucrose, arabinose, inositol and rafinose. The isolate was found to be motile, hydrolyse starch, reduce nitrate but did not utilise urea neither degraded gelatine. The isolate could not withstand salinity and grew better in slightly acid to neutral pH. It was observed that the isolate was not pathogenic by not degrading blood cells when grown in blood agar (Table 4.6). The molecular identification of the isolates using genetic sequence of the 16SRNA

primer confirmed the isolates to be *Sphingobium yanoikuye* with 91.55% similarities (Table 4.7).

Table 4.6: Biochemical Characterisation of selected L-asparaginase producers isolated from soil rhizosphere.

Isolate code	**RSSMO(b)5**	**RSSAI(b)3**	RSSMO(a)**4**	**RSSKS(a)4**	RSSKS(b)**1**	**RSSAB(b)2**
Galactose	-	+	-	-	+	+
Maltose	-	+	+	-	+	-
Mannitol	-	+	+	+	-	-
Lactose	-	-	+	-	+	-
Glucose	+	+	+	+	+	-
Sucrose	-	+	-	+	-	-
Arabinose	-	-	-	+	-	+
Fructose	+	+	+	+	+	+
Inositol	-	+	+	-	-	+
Raffinose	-	+	+	-	-	+
Starch Hydrolysis	+	+	+	+	+	+
Motility	_	+	+	+	+	+
nitrate ultilization	-	-	+	+	-	+
Gram staining	+	-	+	_	-	+
Gelatine	-	+	-	+	-	+
pathogenicity	-	+	+	+	-	-
Pigment	Pink	golden	-	-	-	-
Salinity	-	-	-	-	-	-
urease	-	-	-	-	-	-
pH Tolerance	Slightly acidic	acidic	Slightly acidic	acidic	Slightly acidic	Slightly acidic

Keys

+ positive

- Negative

Table 4.7: Molecular Identification of Selected L-asparaginase producing Bacteria

Isolate codes	Phenotypic identification	% Similarities	Assession numbers
RSSMO(b)5	*Amycolatopsis japonica*	89.50	NA
RSSMO(a)4	*Stenotrophomonas pavanii*	94.55	MN658473
RSSAI(b)3	*Actinomycetales bacterium*	89.52	NA
RSSKS(a)4	*Sphingobacterium caeni*	91.89	MN658474
RSSAB(b)2	*Paenibacillus cineris*	94.60	MN658472
RSSKS(b)1	*Sphingobium yanoikuyae*	91.55	MN658471

Isolate RSSAI(b)3 was observed to be Gram negative rods under the microscope. It was degraded by potassium hydroxide, observed to be motile, utilised starch, gelatin but not urea. It degraded nitrate, did not withstand high salinity but grew well in acidic medium (Table 4.6). It can utilise both mono and di- saccharides sugars tested except lactose and arabinose. It was identified molecularly by 16S rDNA gene sequence and was confirmed to be *Actinomycetal bacterium* when blasted on NCBI gene sequence (Table 4.7).

Isolate RSSAB(b)2 cells were observed to be Gram positive, tiny rod cells, which occurred separately when viewed under microscope. The cells were not degraded by KOH. The isolate could not degrade urea, and did not grow under saline conditions. It grew well in slightly acidic or neutral pH. RSSAB(b)2 was motile, reduced nitrate to nitrite and hydrolysed starch and gelatin. It was also observed that the RSSAB(b)2 showed no level of pathogenecity as it was unable to haemolyse blood when cultured on blood agar. The isolate had the ability to utilise sugars like galactose, arabinose, fructose, inositol and raffinose but was unable to utilise maltose, mannitol, lactose, glucose and sucrose (Table 4.6). The isolate was further identified molecularly and it was confirmed to be *Paenibacillus cineris* (94.60%) when blasted on NCBI gene sequence and maximum likelihood comparism was done (Table 4.7).

Isolate RSSKS(a)4, When viewed microscopically, it was observed to be gram-negative rod with no spore present. The cell wall of the colonies was degraded when mixed with potassium hydroxide. The biochemical studies of the colonies revealed that, the isolates grew well in acidic medium, was not able to utilise urea did not grow well in saline medium. The colonies showed some level of pathogenicity, degraded gelatin, reduced nitrate, hydrolysed starch and was motile. RSSKS(a)4 could utilise mannitol, glucose, sucrose, arabinose and fructose but did not utilise galactose, maltose, lactose, inositol and raffinose (Table 4.6). The isolate was confirmed to be *Shigobacterium caenis* after a blast search through the gene bank of NCBI and the phylogenetic analysis by using maximum like hood companism with the gene sequence of 16S DNA of *Shigobacterium caenis* show 91.89% similarity (Table 4.7).

4.4. Physiological studies of the selected L-asparaginase producers

In Figure 4.2, Tryptone glucose medium was the best medium that supported the growth of all the identified isolates, followed by M9 medium. The medium that least supported the growth of the isolates was Glycerol Asparagine medium. It was also observed that TGY supported the growth of all the isolates in which *Actinomycetal bacterium* showed the highest growth with value of 2.43 nm.The highest growth of *Amycolatopsis japonica* was noticed when grown in TGY, followed by M9, SC, MCD and the lowest growth was in GA. The highest growth of *Actinomycetal bacterium* was in TGY, followed by M9, MCD and the lowest was in GA. *Stenotrophomonas pavani* showed good growth in TGY followed by M9, MCD and the showed the lowest growth in GA followed by SC. *Shigobacterium caenis* showed growth in TGY followed by SC, M9 and lower growth in GA medium followed by MCD. The highest growth of *Sphingobium yanoikuyae* was noticed in TGY followed by SC, M9 and MCD. TGY supported the growth of *Paenibacillus cineris*, followed by SC and M9.

Also, TGY supported the growth of Actinomycetal bacterium, *Stenotrophomonas pavani*, *Shigobacterium caenis*, *Sphingobium yanoikuyae* and did not support the growth of *Paenibacillus cineris* and *Amycolatopsis japonica*. MCD showed the highest growth support for *Amycolatopsis japonica*, *Stenotrophomonas pavani Shigobacterium caenis* but did not support the growth of *Sphingobium yanoikuyae*, *Actinomycetal bacterium* and *Paenibacillus cineris*. M9 medium supported the growth of *Actinomycetal bacterium*, *Sphingobium yanoikuyae*, *Paenibacillus cineris Stenotrophomonas pavani* but showed little support for the growth of *Amycolatopsis japonica* and *Shigobacterium caenis.* SC was found to be the best growth supporters for *Sphingobium yanoikuyae* with 2.07 nm, followed by *Actinomycetal bacterium*, *Shigobacterium caenis* and *Paenibacillus cineris* and less supported the growth of *Stenotrophomonas pavani* and *Amycolatopsis japonica.* GA showed insignificant growth support for all the studied isolates.

Studying the influence of various media on L-asparaginase production from the isolates revealed that, Tryptone Glucose Yeast agar (TGY), followed by M9 medium mostly encouraged the production of L-asparaginase from all the isolates while SC, GA and MC-DOX medium least supported the production of L-asparaginase (L-asp) from all the Isolates. The highest (L- asp) production with

enzyme activity of 0.86 U/mL and 0.75 U/mL was achieved when the enzyme was produced from *Sphingobium yanoikuyae* in M9 and TGY medium respectively while the lowest enzyme activity of 0.01 U/mL was observed in GA medium. No L-asp activity was found from all the isolates studied when MC-DOX medium was used for production (Figure 4.3).

Overall, increased growth and L-asparaginase produced was recorded for *Amycolatopsis japonica*, *Actinomycetal bacterium* and *Stenotrophomonas pavani* using TGY and M9 medium. *Sphingobacterium caeni* recorded a similar trend in both (TGY and M9) medium but recorded a commensurate production in M9 medium. *Sphingobium yanoikuyae* and *Paenibacillus cineris* recorded increased growth in Starch casein medium but commensurate L-asparaginase production was recorded in TGY and M9 medium.

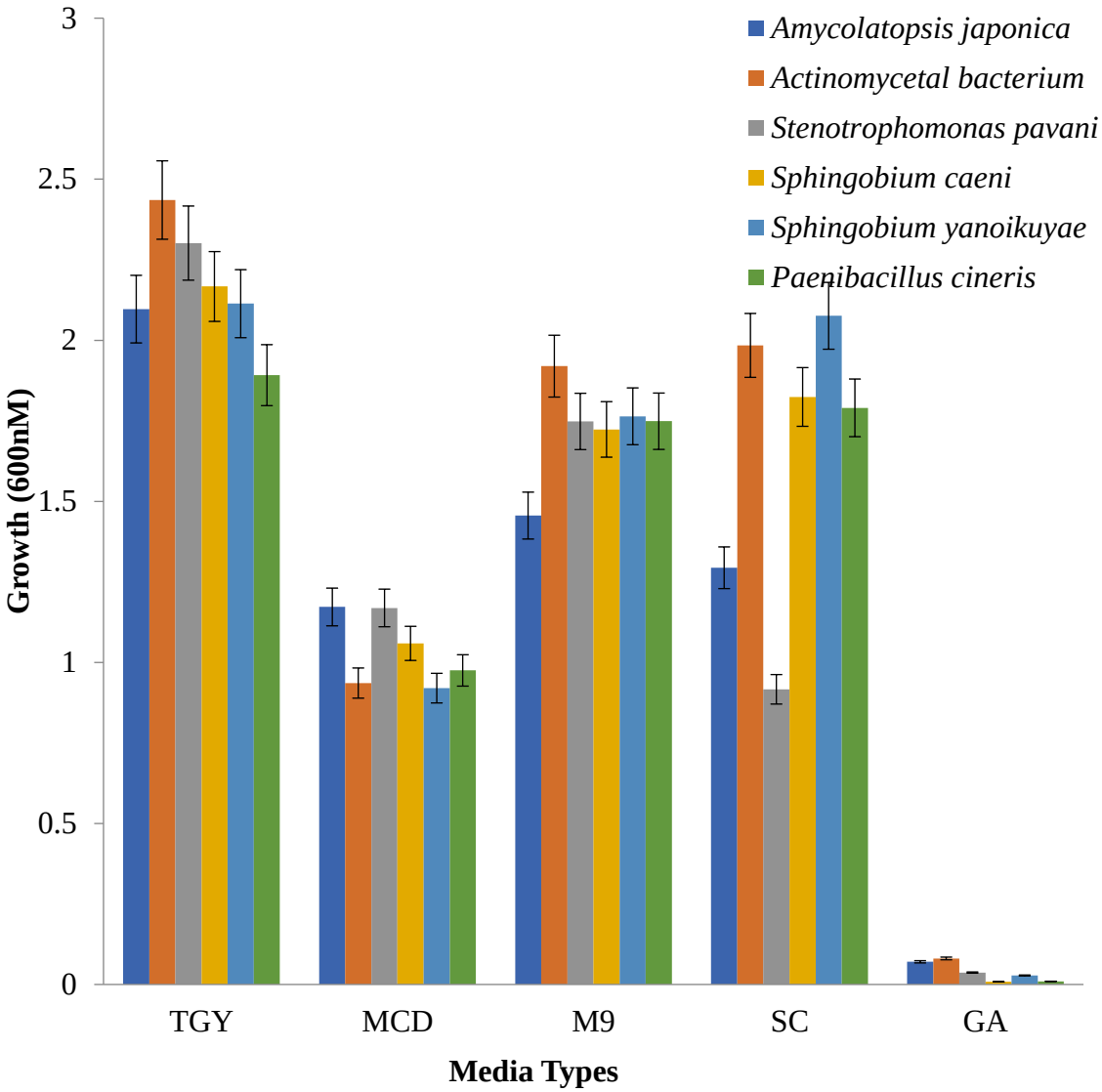


Figure 4.2: Effect of growth media on selected L-asparaginase producers

Keys

TGY: Tryptone glucose yeast medium

MCD: Mc-Dox medium

M9: Modified M9 medium

SC: Starch Casein medium

GAA: Glycerol Asparagine

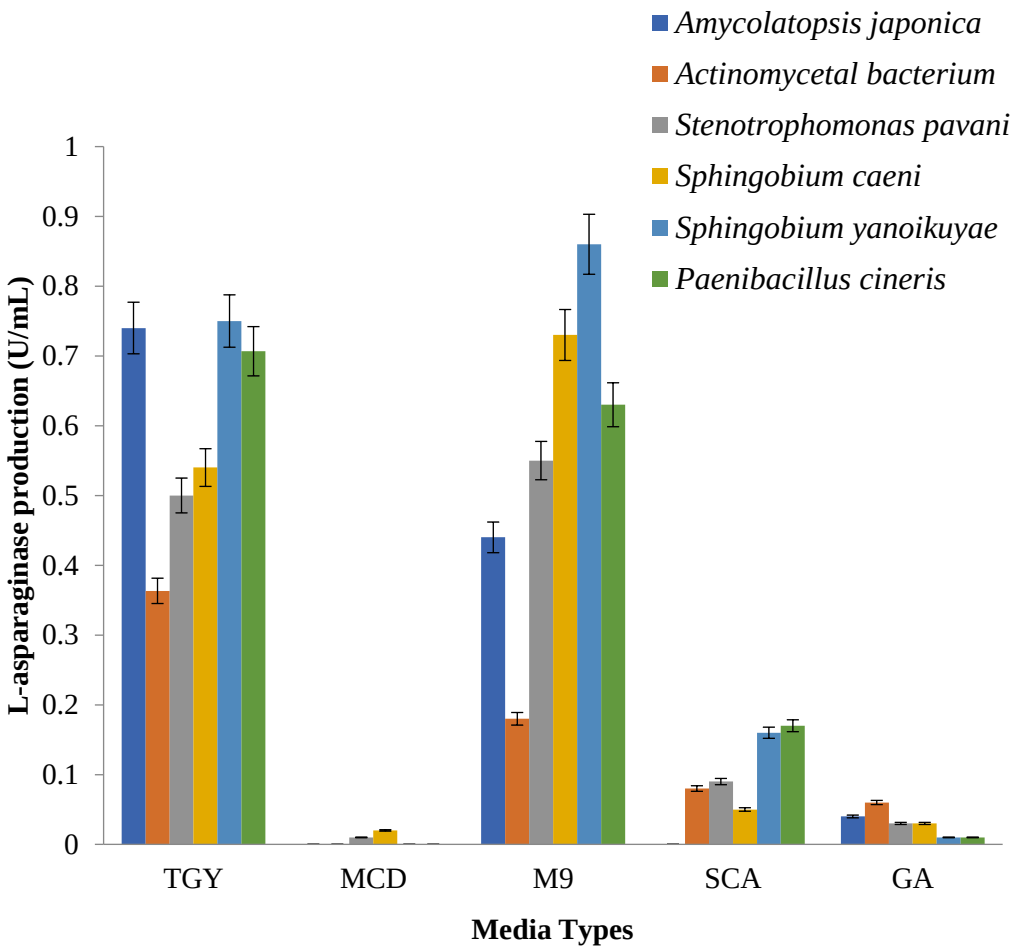


Figure 4.3: Effect of growth media on L-asparaginase production from selected L-asparaginase producers.

Keys

TGY: Tryptone glucose yeast medium

MCD: Mc-Dox medium

M9: Modified M9 medium

SC: Starch Casein medium

GAA: Glycerol Asparagine

The effect of different temperature on the growth and L-asparaginase production from selected isolates is given in Figure 4.4 and 4.5. The figures revealed that the highest growth occurred between the temperature ranges of 25^{o}C to 35^{o}C in all the isolates and gradually decreased from 40^{o}C to 50^{o}C. The highest growth (1.83 nm) was observed from *Actinomycetal bacterium* at 30^{o}C while the lowest growth of (0.004 nm) was from *Amycolatopsis japonica* at 50^{o}C. *Actinomycetal bacterium* was able to grow well at 25^{o}C to 45^{o}C before a sudden reduction at 50^{o}C. *Amycolatopsis japonica* showed the highest growth (0.37 nm) at 30^{o}C and the lowest growth (0.004 nm) at 50^{o}C. *Stenotrophomonas pavani* showed the highest growth (1.73 nm) at 35^{o}C and the lowest growth (0.06 nm) at 50^{o}C. 25^{o}C best encouraged the growth (1.26 nm) of *Shigobacterium caenis* and the lowest growth (0.28nm) was noticed at 50^{o}C. The best growth (1.33 nm) and (1.06 nm) for *Sphingobium yanoikuye* was observed between 30^{o}C and 35^{o}C respectively, after which there was drastic reduction, to no growth (0.23 nm, 0.05 nm) as the temperature increases. The highest growth (0.94 nm) of *Paenibacillus cineris* was noticed at 35^{o}C and the lowest (0.02 nm) at 50^{o}C.

Studying the L- asparaginase (L-asp) production at different temperatures (Fig 4.5), the highest L-asparaginase production was noticed from *Paenibacillus cineris* at 35^{o}C and the lowest production (0.I9 U/mL) was noticed from *Shigobacterium caenis* at 30^{o}C. At 45^{o}C and 50^{o}C, no L-asparaginase production was noticed from *Amycolatopsis japonica* and the highest production (0.24 U/mL) was at 35^{o}C. From *Actinomycetal bacterium*, it was observed that the L-asparaginase production increased with increase in temperature, with the highest enzyme (0.77 U/mL) produced at 50^{o}C and no production (0.01 U/mL) at 25^{o}C. *Stenotrophomonas pavani* produced its highest L-asparaginase (0.54 U/mL) at 35^{o}C and the lowest enzyme production (0.13 U/mL) at 25^{o}C and 30^{o}C. *Shigobacterium caenis* showed highest enzyme production (0.10 U/mL) at 35^{o}C and the lowest enzyme production (0.01 U/mL) was at 50^{o}C. *Sphingobium yanoikuyae* gave the highest L-asparaginase production (0.63 U/mL) at 35^{o}C and no enzyme was produced at 45^{o}C.

Overall, a temperature of 25 to 35^{o}C supported high growth and L-asparaginase production by *Amycolatopsis japonica.* Temperature of 25 to 45^{o}C supported highest growth of *Actinomycetal bacterium* but complementary enzyme production

was recorded at 35 to 45^{o}C. A temperature of 30 to 35^{o}C supported highet growth in *Stenotrophomonas pavani* but the enzyme production was at 35 to 45^{o}C. *Sphingobacterium caeni* recorded increased growth at 25 to 45^{o}C but commensurate L-asparaginase production was recorded at 35^{o}C. Temperature of 30 to 35^{o}C supported high growth in *Sphingobium yanoikuyae* but complementary enzyme production was only recorded at 30 to 40 ^{o}C. Increased growth and L-asparaginase produced was recorded for *Paenibacillus cineris* between the temperatures of 35 to 40 ^{o}C.

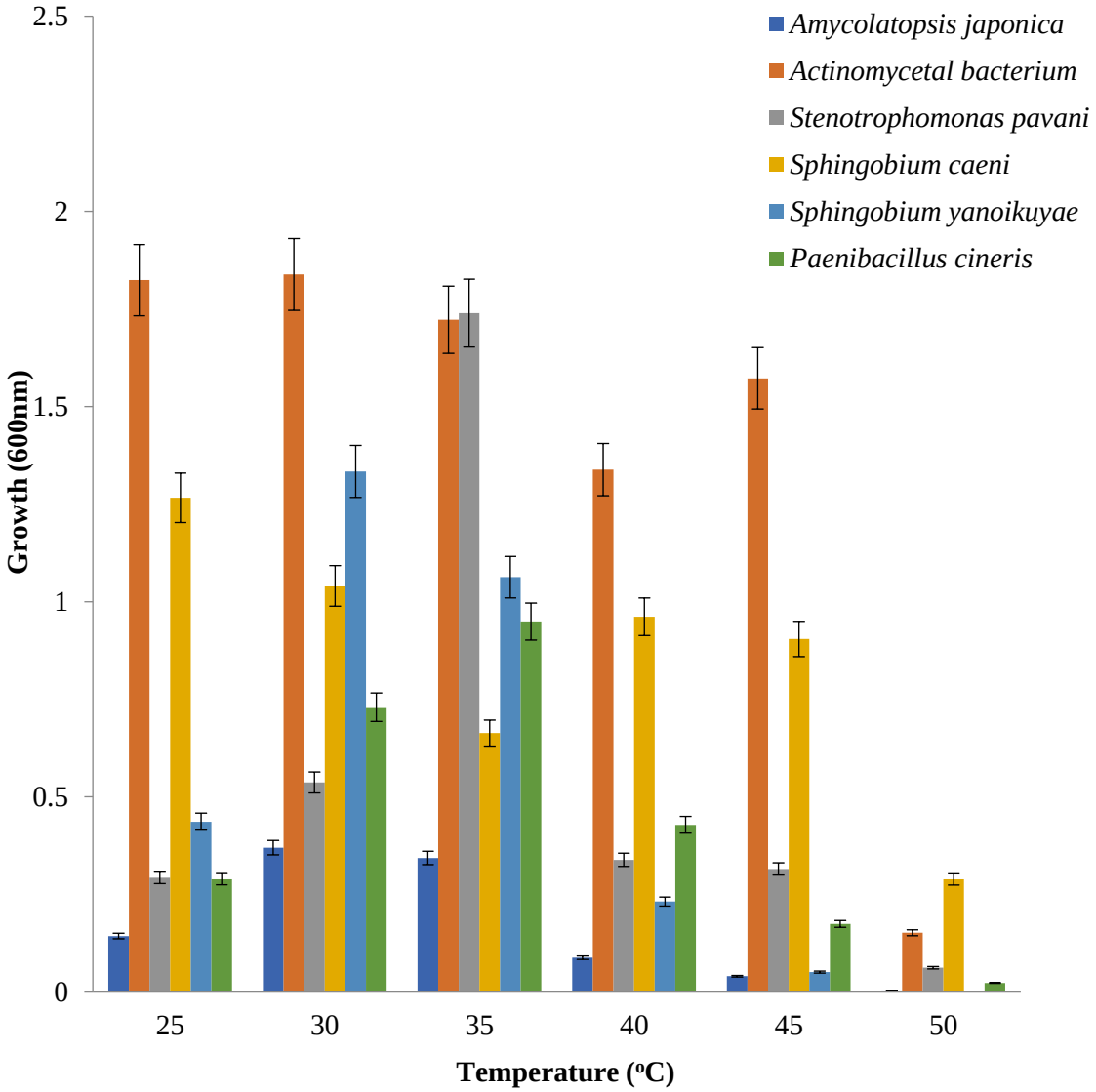


Figure 4.4: Effect of temperature on the growth of selected L-asparaginase producers

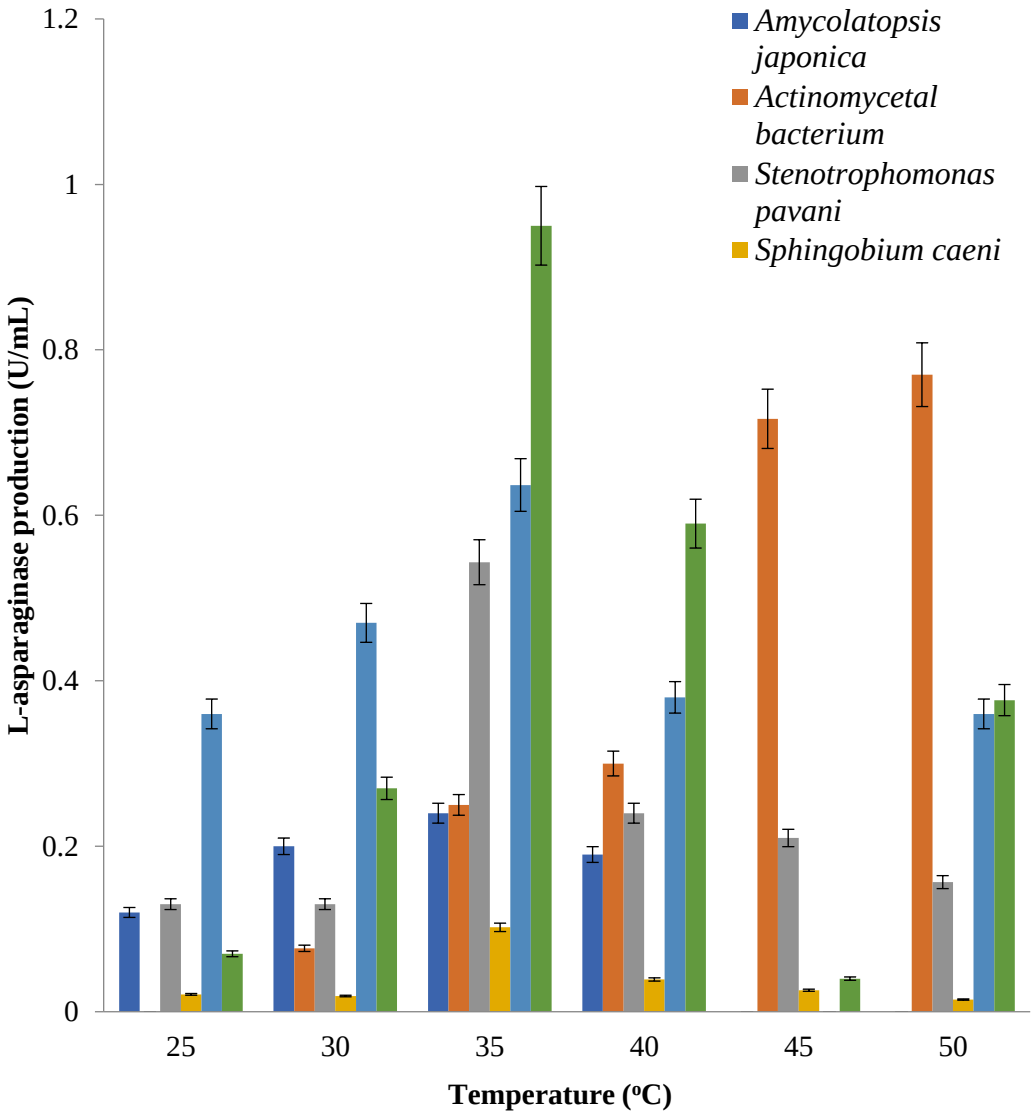


Figure 4.5: Effect of temperature on L-asparaginase production on selected L-asparaginase producers

The effect of pH on the growth and L-asparaginase production by the selected isolates was also recorded in Fig 4.6 and 4.7. The optimum growth of all the isolates was at the pH range of 5 to 8 and minimal at pH 4, 9 and 10 except *Actinomycetal bacterium* that had it optimum growth (2.02 nm) at pH 4.

The optimum pH for the growth (1.96 nm) of *Amycolatopsis japonica* was at pH 7 and the lowest growth (0.04 nm) was at pH 10 (Fig 4.12). There was decrease in its growth after pH 8, *Actinomycetal bacterium* showed optimum growth (2.02 nm) at pH 4 and the minimum growth (0.75 nm) at pH 9. There was significant growth between pH 4 and 8. The highest growth (1.83 nm) of *Stenotrophomonas pavani* was at pH 7 and the lowest growth (0.10 nm) was at pH 4. Increase in the growth was noticed as the pH increased and good growth was achieved between pH 6 and 8. *Shigobacterium caenis* showed higher growth between pH 6 and 8 with the optimum growth (1.05 nm) at pH 6 and lowest growth (0.10 nm) at pH 10. The optimum growth (1.72 nm) of *Sphingobium yanoikuyae* was at pH 6 and lowest growth with (0.007 nm) at pH 4. There was no growth of the isolate from pH 10. *Paenibacillus cineris* gave the highest growth (1.62 nm) at pH 5 and the lowest growth (0.04 nm) at pH 4. Varying the pH of the culture medium for L-asparaginase production by these organisms, it was observed that pH 7 showed the highest support for L-asparaginase production with the enzyme activity (0.88 U/mL) by *Amycolatopsis japonica* and the least support for L-asparaginase production was seen at pH 10 by *Sphingobium yanoikuyae* with enzyme activity of (0.05 U/mL). *Amycolatopsis japonica*'s highest L-asparaginase production was achieved at pH 7 with enzyme activity of 0.88U/mL and the minimum enzyme production was at pH 9 and 10 with enzyme activity of 0.08 U/mL and 0.1 U/mL, respectively. *Actinomycetal bacterium* gave highest L-asparaginase production at pH 8 with the enzyme activity of 0.7 U/mL and the least production at pH 4 with the enzyme activity of 0.19 U/mL. A pH of 6 gave the highest support for L-asparaginase production by *Stenotrophomonas pavani* with the enzyme activity (0.28 U/mL) and the lowest enzyme production (0.08 U/mL) was found at pH 10. *Shigobacterium caenis* gave highest L-asparaginase production at pH 4 with the enzyme activity of 0.54 U/mL and the lowest production (0.12 U/mL) at pH 6. A pH of 6 gave the highest support for L-asparaginase production by *Sphingobium yanoikuyae* with enzyme activity of 0.05 U/mL and the pH 9 and 10 with enzyme activity of 0.03

U/mL and 0.05 U/mL, respectively. The optimum L-asparaginase production by *Paenibacillus cineris* was noticed at pH 8 with enzyme activity of 0.62 U/mL and the least L-asparaginase production was at pH 9 with enzyme activity of 0.08 U/mL.

Overall, a pH of 4 to 8 supported high growth and L-asparaginase production by *Amycolatopsis japonica. Actinomycetal bacterium* recorded a similar trend at pH 4 to 8 but recorded a commensurate L-asparaginase production at pH 6 to 8. A pH of 6 to 8 supported high growth of *Stenotrophomonas pavani* but complementary enzyme production was recorded at pH 5 to 8. A pH of 6 to 9 supported high growth of *Sphingobacterium caeni* but complementary enzyme production was recorded at pH 4 to 5. *Sphingobium yanoikuyae* recorded increased growth at the pH of 5 to 7 but commensurate L-asparaginase production was recorded at pH 5 to 7. pH 5 supported high growth in *Paenibacillus cineris* but complementary enzyme production was only recorded at the pH of 5 to 8.

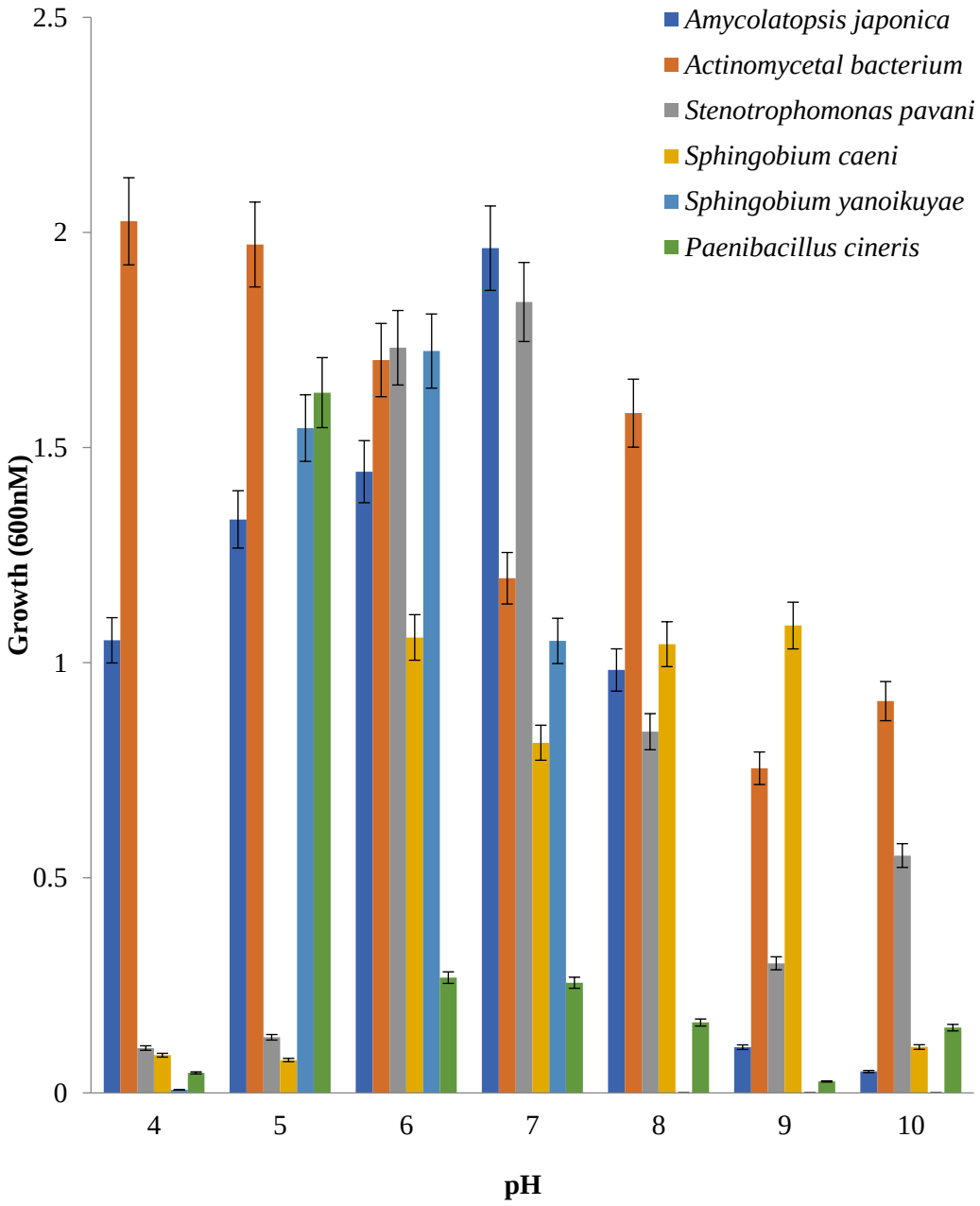


Figure 4.6: Effect of pH on the growth of selected L-asparaginase producers

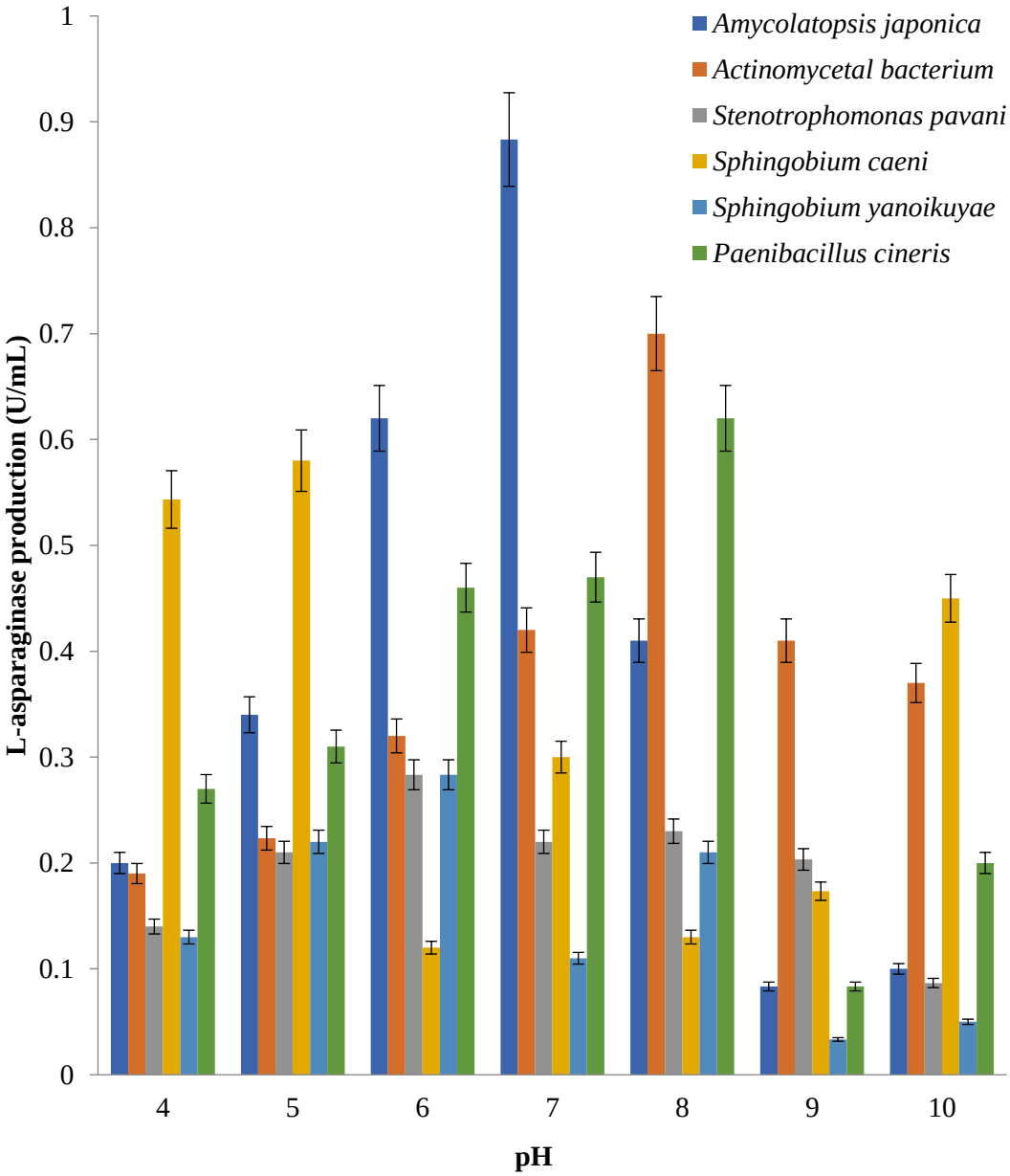


Figure 4.7: Effect of pH on L-asparaginase production from selected L-asparaginase producers

The effect of different innoculum sizes on the growth of the selected isolated revealed that the growth of isolates depended on their quantity in the cultivation medium. The growth of all the isolates was favoured with 1.5 x 10^8 CFU/mL. The highest growth (1.80 nM) was observed when 3 x 10^8 CFU/mL was used and the lowest growth (0.03 nm) was from *Sphingobium yanoikuyae* when 9 x 10^8 CFU/mL was used. The growth of *Amycolatopsis japonica* and *Sphingobium yanoikuyae* decreased with increase in the inoculum sizes, while for *Paenibacillus cineris* the growth increase with increasing inoculum sizes (Figure 4:8).

The effect of inoculum sizes on the L-asparaginase production is as shown in figure 4:9. It was observed that the highest L-asparaginase production was achieved when 6 x 10^8 CFU/mL, of *Actinomycetal bacterium* was used for production and the lowest activity was achieved when 9 x 10^8 CFU/mL of the same isolates was used. The L-asparaginase production by *Amycolatopsis japonica* was noted to be decreasing with increase in inoculum size. The highest enzyme production (0.67 U/mL) was achieved when 1.5 x 10^8 CFU/mL was used and the least production (0.06 U/mL) was achieved when 9 x 10^8 CFU/mL was used. Likewise for *Paenibacillus cineris*, the highest and least production (0.99 U/mL) and (0.42 U/mL) was achieved when 1.5 x 10^8 CFU/mL and 12 x 10^8 CFU/mL were used. The highest L-asparaginase production from *Stenotrophomonas pavani* was achieved in 3 x 10^8 CFU/mL with enzyme activity of 1.12 U/mL and the least production was 0.44 U/mL. The L-asparaginase production by *Sphingobacterium caenis* was noted to be increasing with increase in inoculum size up to 6 x 10^8 CFU/mL after which there was decrease in the production. The highest enzyme production (1.02 U/mL) was achieved when 6 x10^8 CFU/mL was used and the lowest production (0.7 U/mL) was achieved when 9 x 10^8 CFU/mL was used. *Sphingobium yanoikuyae*, gave the highest and lowest enzyme production of 0.99 U/mL and 0.42 U/mL, when 1.5 x 10^8 CFU/mL and 12 x 10^8 CFU/mL was used for L-asparaginase production. The highest L-asparaginase production from *Paenibacillus cineris* was achieved in 12 x 10^8 CFU/mL with enzyme activity of 1.42 U/mL and the lowest activity was 0.6 U/mL in 9 x 10^8 CFU/mL.

Overall, increased growth was recorded for *Amycolatopsis japonica* when 1.5 x 10^8 to 3 x 10^8 CFU/mL was used but commensurate L-asparaginase production was in a medium with 1.5 x 10^8 CFU/mL. *Actinomycetal bacterium* recorded highest

growth when 3 x 10^8 CFU/mL was used, but the optimum L-asparaginase yield was in a medium with 3 x 10^8 CFU/mL and 1.5 x 10^8 CFU/mL. Increased growth and L-asparaginase produced was recorded for *Stenotrophomonas pavani and Sphingobacterium caeni* when 1.5 and 3.0 x 10^8 CFU/mL and 6.0 x 10^8 CFU/mL was used respectively. Also, *Sphingobium yanoikuyae* recorded its maximum growth and L-asparaginase production when 1.5 – 6.0 x 10^8 CFU/mL was used.

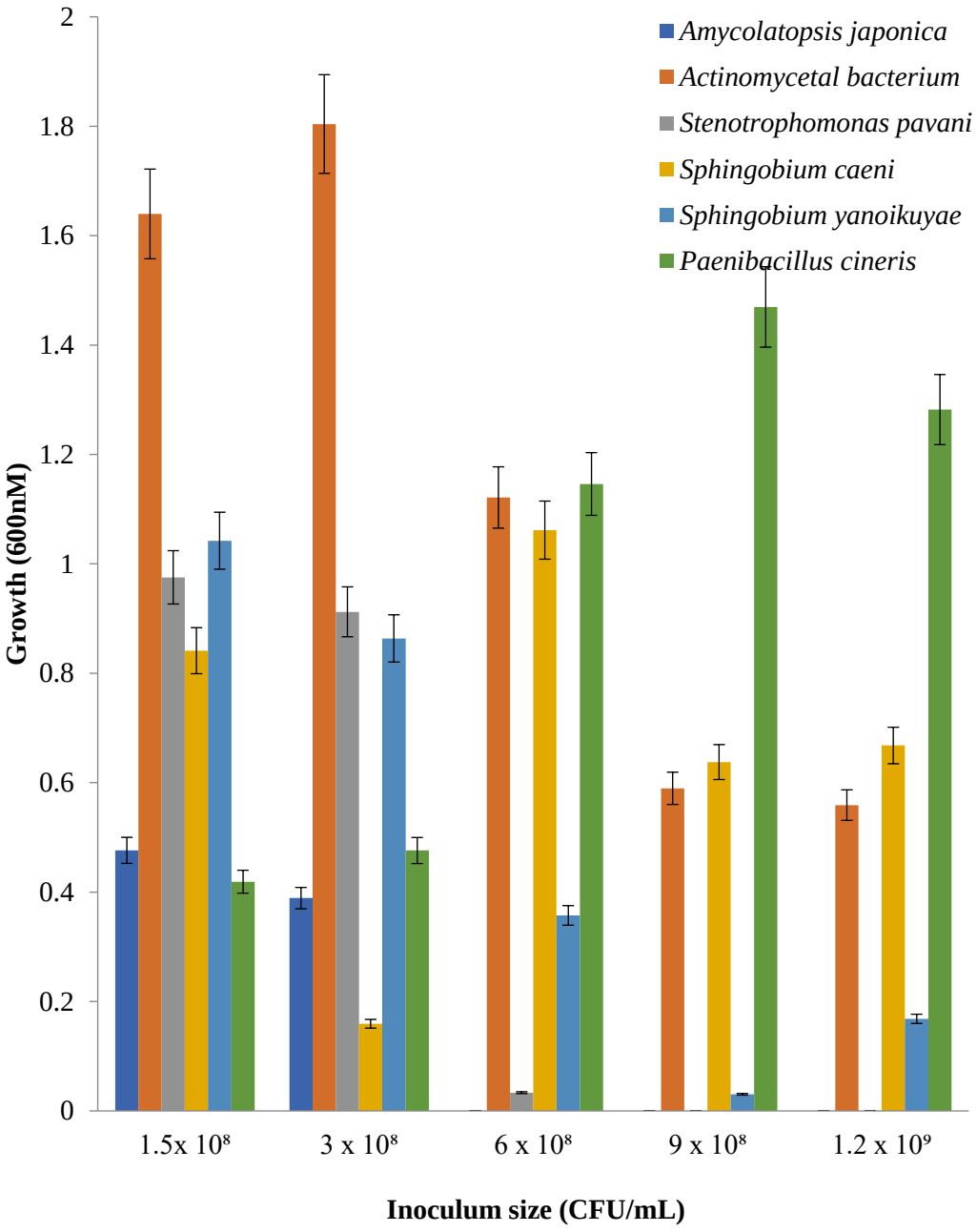


Figure 4.8: Effect of inoculum sizes on the growth of selected L-asparaginase producers

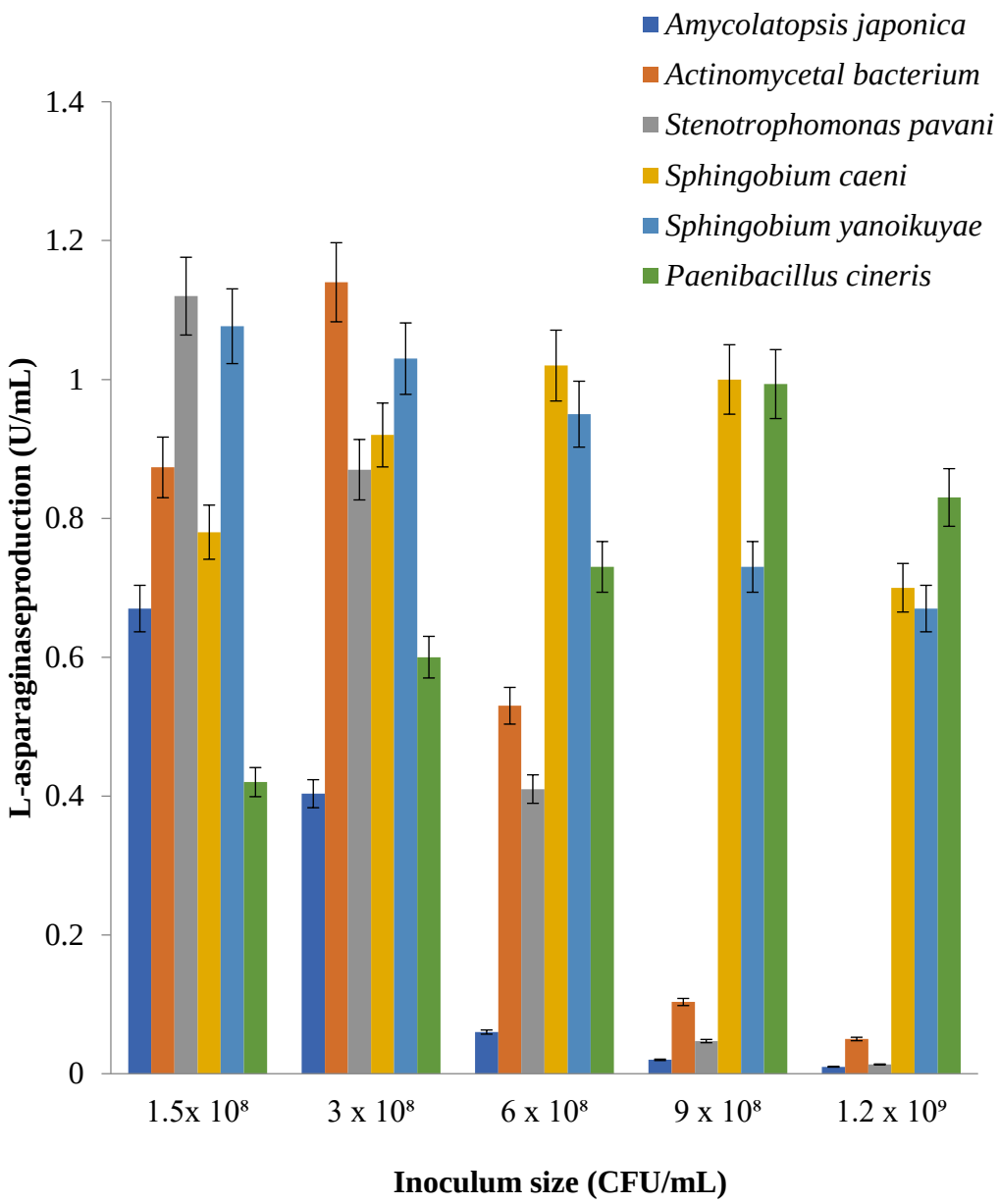


Figure 4.9: Effect of inoculum sizes on L-asparaginase production from selected L-asparaginase producers

After the culture medium was incubated at different days intervals (day 3 to 14), the effect of the incubation periods on the growth and L-asparaginase producthe selected isolates is shown in Figure 4.10 and 4.11. From the result, the highest growth (1.80 nm) was found at the day 3 by *Shigobacterium caenis* (Fig 4.10). The optimum growth of the *Amycolatopsis japonica* was attained at day 7 and the lowest growth was at day 14. There was increase as the growth progressed up to 10 days after which there was a gradual decrease in the growth. *Actinomycetal bacterium* showed significant growth throughout the days of the incubation with the highest growth at day 4 and the lowest growth at day 5. Day 10 gave the highest growth support for *Stenotrophomonas pavani* and the lowest growth was seen at the day 14. The highest growth of *Shigobacterium caenis* was attained at day 3 and the lowest growth at day 10. *Sphingobium yanoikuyae* attained the highest growth at day 14, and the lowest growth at day 3. It was observed that the growth of *Sphingobium yanoikuyae* increased with increase in period of incubation. *Paenibacillus cineris* attained the highest growth at day 12 and the lowest at day 3.

Also for the L-asparaginase production (Fig 4.10), the highest L-asparaginase production was achieved at day 7 with enzyme activity of 2.42 U/mL by *Paenibacillus cineris* and lowest L-asparaginase production was at day 3 and 10 with enzyme activity of 0.17 U/mL and 0.16 U/mL by *Sphingobium yanoikuyae* and *Sphigobacterium caenis* respectively. The highest L-asparaginase production from *Amycolatopsis japonica* was achieved at day 7 with activity of 1.97 U/mL and the lowest was achieved at day 14 with enzyme activity of 0.67 U/mL. There was increase in the enzyme activity from day 3 to 7 and a gradual decrease from day 10 to 14. *Actinomycetal bacterium* showed highest L-asparaginase production at day 7 with enzyme activity of 1.31 U/mL after which there was inconsistency in its activity from day 10 to 14. The optimum L-asparaginase for *Stenotrophomonas pavani* was noticed at day 10 with enzyme activity of 2.33 U/mL and the lowest production at day 3 with enzyme activity of 0.67 U/mL. An increase in production as the days increased was also noticed. *Sphigobacterium caenis* achieved it highest L-asparaginase production at day 7 with enzyme activity of 1.38 U/mL at day 14 and the lowest production was at day 10 with enzyme activity of 0.16 U/mL. *Sphingobium yanoikuyae* attained its

optimum L-asparaginase production at day 10 with enzyme activity of 2.01 U/mL and the lowest L-asparaginase production was noticed at day 3 with enzyme activity of 0.17 U/mL. There was a gradual increase in the production as the days increased. A decrease in the production was also noticed after 10 days of production. *Paenibacillus cineris* gave its highest L-asp production at day 7 with enzyme activity of 2.42 U/mL and the lowest production at day 3 with enzyme activity of 1.37 U/mL. Increase in the L-asparaginase production as the day's progresses was also noticed.

Overall, Day 3 to 7 supported high growth and L-asparaginase production by *Amycolatopsis japonica.* Also *Actinomycetal bacterium* recorded its maximum growth and L-asparaginase production between day 3 and 14. Between day 5 and 10 supported high growth of *Stenotrophomonas pavani* but complementary enzyme production was recorded between day 5 and 14. *Sphingobacterium caeni* recorded its maximum growth between day 3 and 7 but the complementary enzyme production was recorded between day 5 and 7.

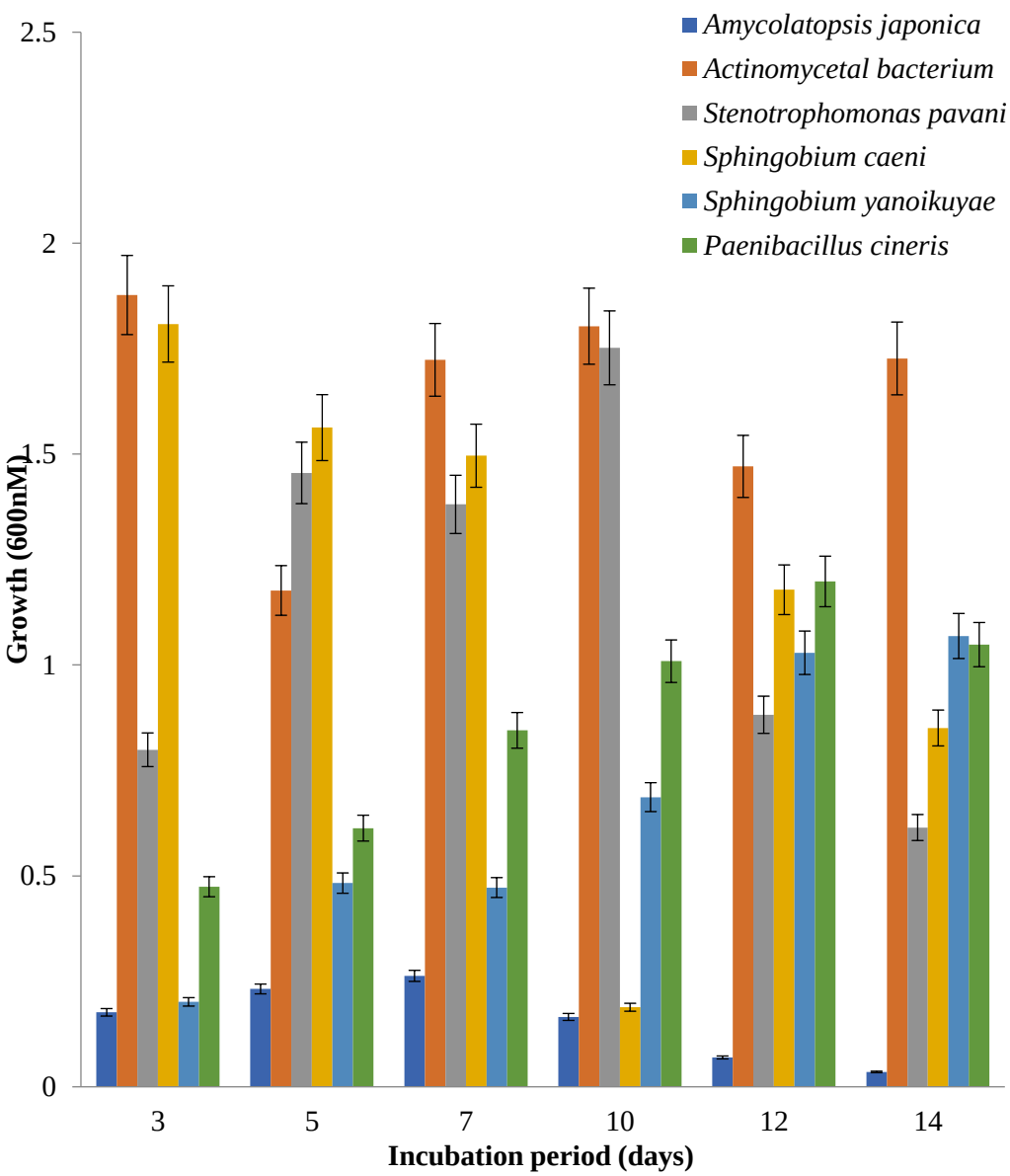


Figure 4.10: Effect of incubation period on the growth of selected L-asparaginase producers

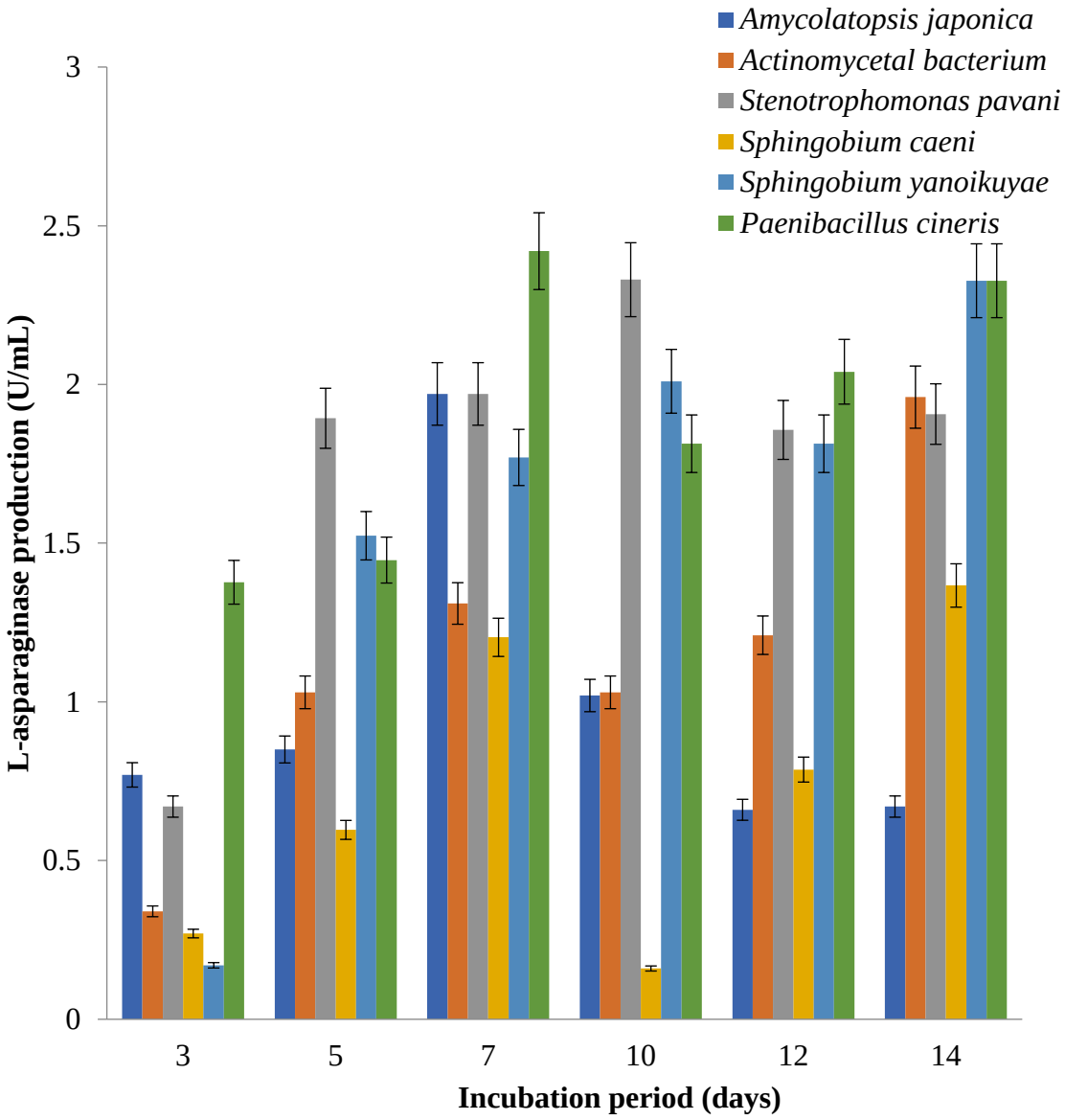


Figure 4.11: Effect of incubation period on L-asparaginase production from selected L-asparaginase producers

The effect of carbon sources on the growth and L-asparaginase production by selected isolates is shown in Figures 4.12 and 4.13. Growth of *Amycolatopsis japonica* was highest in the presence of maltose and CMC less supported its growth. Fructose is the best growth enhancer for *Actinomycetal bacterium*, followed by sucrose and the least supporter was CMC. Starch showed the best support for the growth of *Stenotrophomonas pavani*, followed by fructose and sucrose. The least growth of the isolate was seen on lactose. *Shigobacterium caenis* exhibited the highest growth in the presence of groundnut shell, fructose and maltose and the lowest yield was seen in the presence of glucose. All the carbon sources supported the growth of *Sphingobium yanoikuyae* with the highest growth support from starch, followed by groundnut shell, and sucrose. The least supporter of *Sphingobium yanoikuyae* was fructose with growth value of 0.68 nm followed by maltose. Maltose best supported the growth of *Paenibacillus cineris*, followed by sucrose. lactose, CMC and glucose did not support the growth of these organisms.

The optimum L-asparaginase production by *Amycolatopsis japonica* was achieved in the presence of maltose, followed by groundnut with the enzyme activity of 0.23 U/mL and 0.13 U/mL respectively and the lowest production was achieved in the presence of glucose with enzyme activity of 0.03 U/mL. In the presence of glucose, the maximum L-asparaginase production was achieved by *Actinomycetal bacterium* and the lowest production was achieved in the presence of sucrose. No L-asparagnase production was achieved in the presence of starch and CMC. The L-asparaginase production by *Stenotrophomonas pavani* was optimum in the presence of glucose, followed by fructose with enzyme activity of 0.33 U/mL and 0.11 U/mL respectively and the lowest L-asparaginase production was seen in the presence of groundnut shell. There was no L-asparaginase production in the presence of starch (0.013 U/mL). L-asparaginase production by *Shigobacterium caenis* was enhanced in the presence of fructose with optimum enzyme activity of 15.13 U/mL followed by starch and lactose (12.38 U/mL and 9.11 U/mL) respectively and the least production in the presence of glucose: there was no L-asparaginase production in the presence of CMC, maltose, groundnut and sucrose by *Shigobacterium caenis*. The optimum L-asparaginase production by *Sphingobium yanoikuyae* was achieved in the presence of starch and fructose with the enzyme activity of 0.423 U/mL and 0.42

U/mL. There was no L-asparaginase production in the presence of maltose by *Sphingobium yanoikuyae*. None of the carbon sources supported L- asparaginase production from *Paenibacillus cineris*.

Overall, increased growth for *Amycolatopsis japonica* was recorded in the presence of maltose, fructose and sucrose, but the commensurate enzyme production was in the presence of maltose. *Actinomycetal bacterium* recorded a similar trend, but recorded a commensurate L-asparaginase production in the presence of glucose, maltose and fructose. *Stenotrophomonas pavani* recorded increased growth in the presence of starch, maltose and sucrose, but recorded maximum L-asparaginase poduction in the presence of glucose. Groundnut shell, fructose and sucrose supported highest growth of *Sphingobacterium caeni* but the commensurate enzyme production was in the presence of fructose and starch. *Sphingobium yanoikuyae* recorded high growth in the presence of starch and groundnut shell, but increased L-asparaginase yield was in the presence of starch and fructose. *Paenibacillus cineris* recorded increased growth and L-asparaginase production in the presence of maltose and fructose.

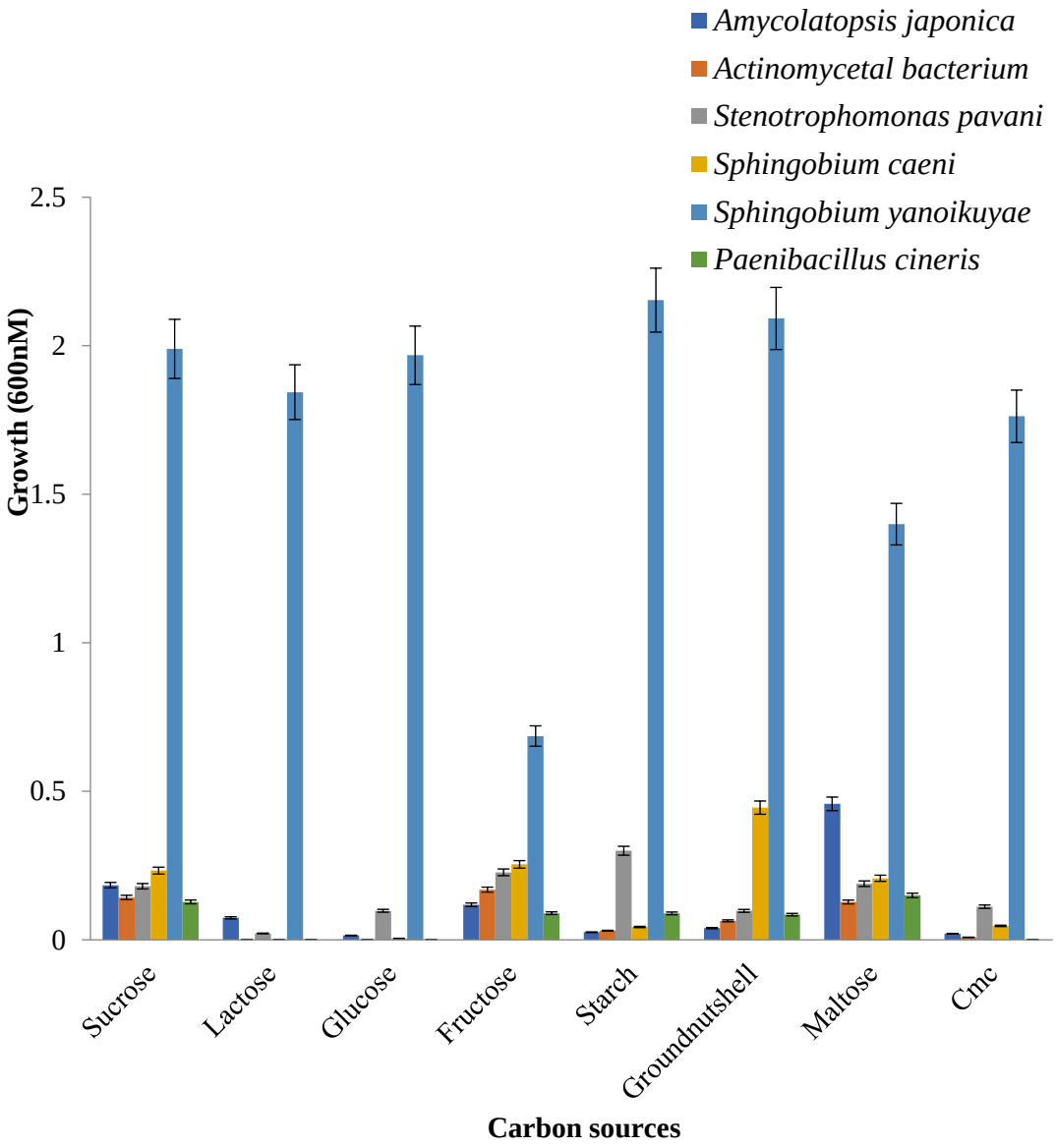


Figure 4.12: Effect of carbon sources on the growth of selected isolates L-asparaginase producers.

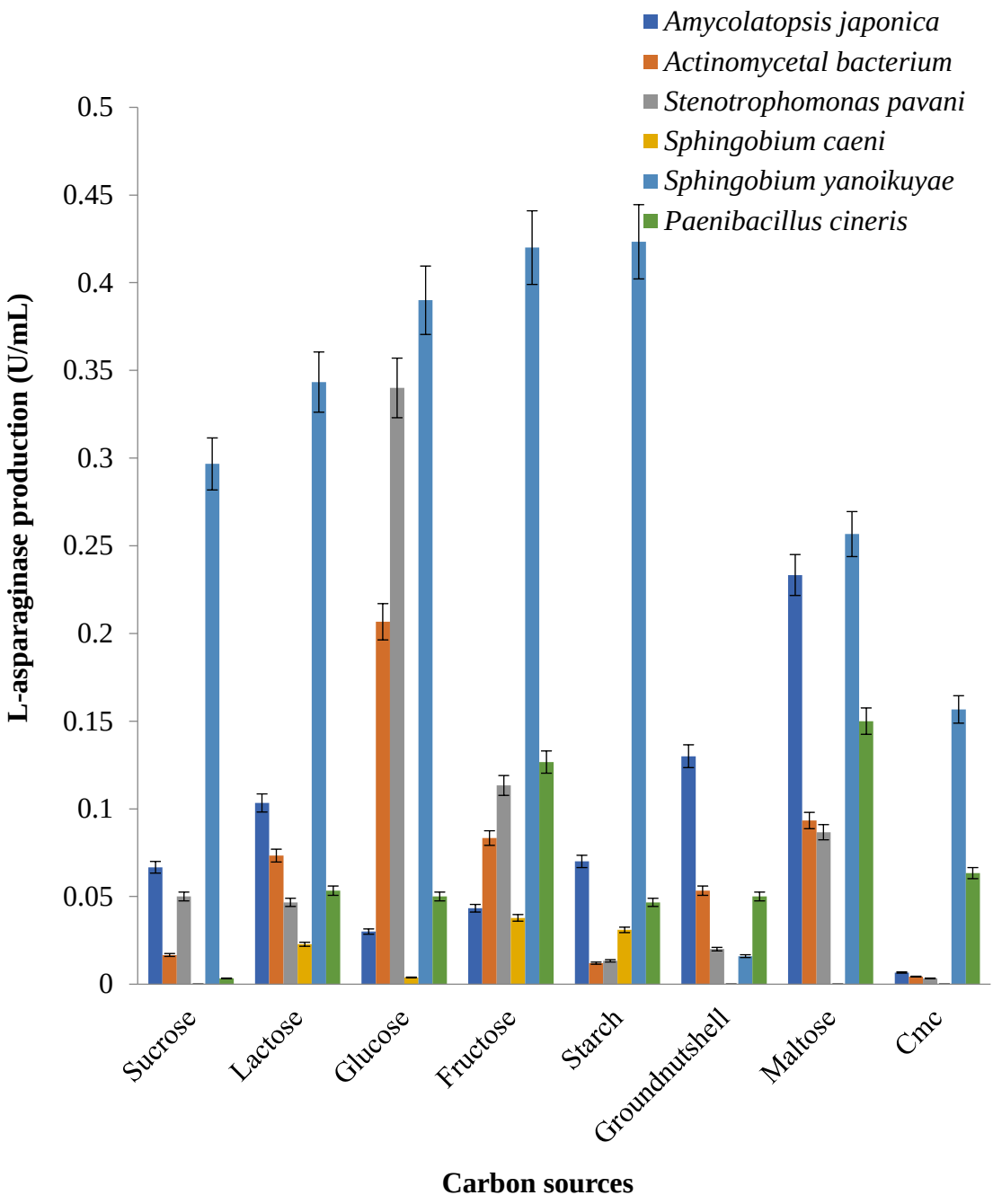


Figure 4.13: Effect of carbon sources on L-asparaginase production from selected L-asparaginase producers

Figures 4.14 and 4.15 showed effect of Nitrogen sources on the growth and L-asparaginase production by selected isolates. Yeast extract was the best growth supporter for *Amycolatopsis japonica*, followed by tryptone and Ammonuim sulphate $(NH)_2SO_4$, least supported the growth of *Amycolatopsis japonica*. The highest growth of *Actinomycetal bacterium* was attained in the presence of Tigernut and the least growth in the presence of tryptone. *Stenotrophomonas pavani* exhibited highest growth in the presence of tigernut and the least growth was seen in the presence of soybean. *Shigobacterium caenis* attained the optimum growth in the presence of tigernut, followed by $(NH)_2SO_4$ and wheat. Soybean least supported the growth of *Shigobacterium caenis*. All the nitrogen tested showed significant growth support for *Sphingobium yanoikuye* with the highest growth achieved in the presence of yeast extract followed by tryptone and $NaNO_3$, the lowest growth was in the presence of $(NH)_2SO_4$. Tigernut was the best supporter of the growth of *Paenibacillus cineris* while tryptone least supported its growth.

On the effect of nitrogen sources on L-aspraginase production, the optimum L-asparaginase production by *Amycolatopsis japonica* was in the presence of tryptone and yeast extract with enzyme activity of (0.39 U/mL and 0.36 U/mL) respectively. There was no significant difference between tryptone and yeast extract. The minimum L- asparaginase production by *Amycolatopsis japonica* was in the presence of wheat and Tigernut (0.03U/mL and 0.033U/mL) respectively. There was no L-asparginase production in the presence of $(NH)_2SO_4$, KNO_3 and $NaNO_3$. The maximum L-asparginase production from *Actinomycetal bacterium* was achieved in the presence of Soybean extract followed by $(NH)_2SO_4$,with enzyme activity of 0.03 U/mL and 0.032 U/mL and the lowest production was in the presence of yeast extract (0.003 U/mL). Soybean extract was the best supporter of L-asparginase production by *Stenotrophomonas pavani* in the enzyme activity of 0.48 U/mL while wheat was the least L-asparaginase supporter with enzyme activity of 0.10 U/mL. Tigernut best supported the production of L-asparginase by *Shigobacterium caenis* with enzyme activity of 0.041 U/mL and the lowest L-asparginase production was in the presence of wheat. No L-asparginase was produced in the presence of tryptone and $NaNO_3$. The maximum L-asparginase production by *Sphingobium*

yanoikuyae was in the presence of tryptone followed by tigernut and yeast extract (0.32 U/mL, O.29 U/mL and 0.28 U/mL). There was no L-asparaginase production in the presence of KNO_3 and $NaNO_3$ by *Sphingobium yanoikuyae*. *Paenibacillus cineris* gave the highest L-asparaginase production in the presence of tryptone and no enzyme was produced in the presence of KNO_3 and $NaNO_3$.

Amycolatopsis japonica recorded high growth in the presence yeast extract, but the optimum L-asparaginase yield was in the presence of Trptone and Yeast extract. Increased growth for *Actinomycetal bacterium* and *Stenotrophomonas pavani* was recorded in the presence of Tigernut, but the commensurate enzyme production was in the presence of Soybean, Ammonnium sulphate and Tigernut. *Sphingobacterium caeni* recorded increased growth in the presence of Tigernut and Maltose, but recorded maximum L-asparaginase production in the presence of Tigernut and Soybean. Yeast extract, Sodium Nitrate and Tryptone supported high growth in *Sphingobium yanoikuyae* but the commensurate enzyme production was in the presence of Tryptone, Yeast extract and Tigernut. Increased growth was recorded for *Paenibacillus cineris* in the presence of Tigernut and Soybean, but the commensurate L-asparaginase produced was in the presence of Tryptone.

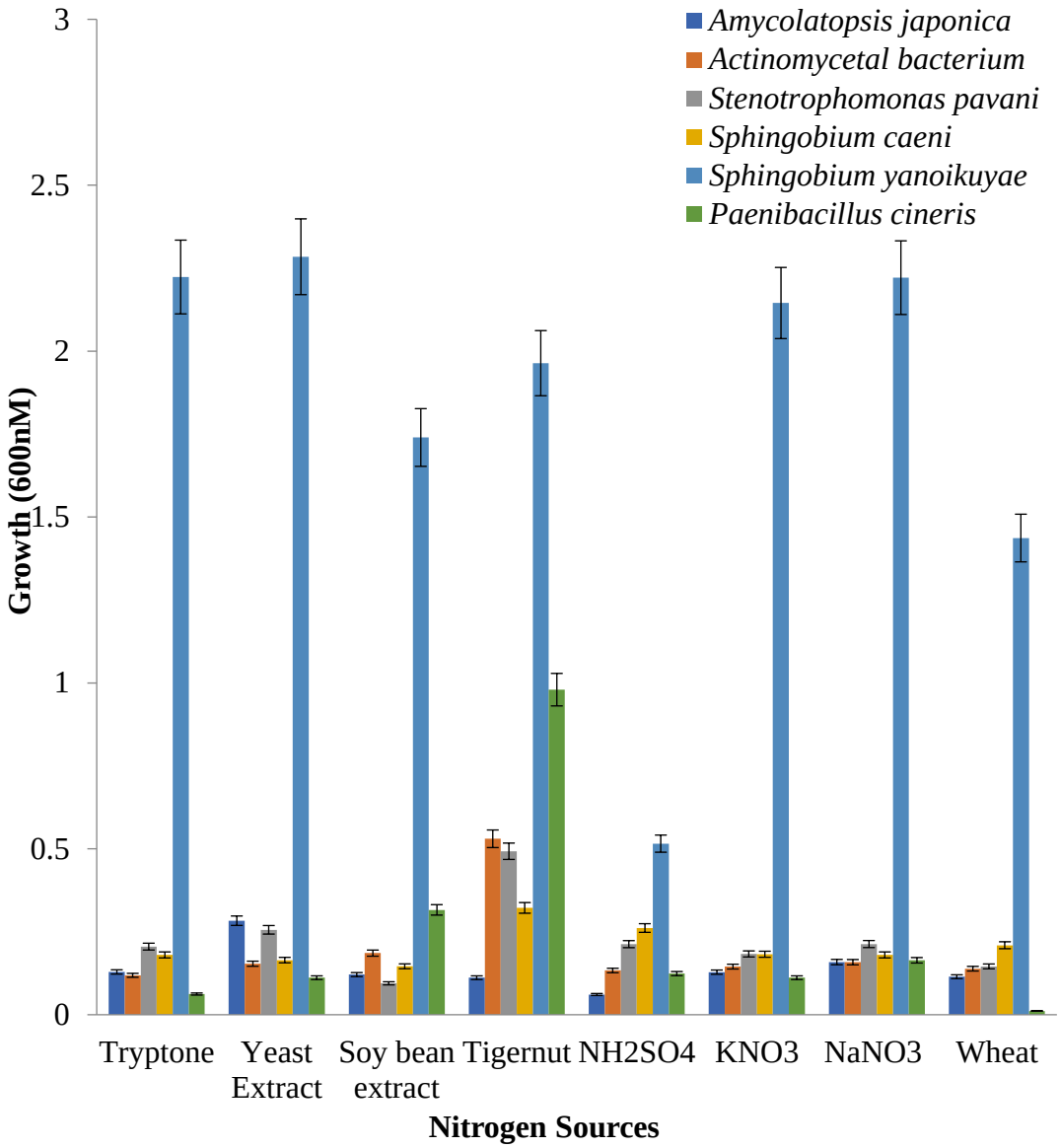


Figure 4.14: Effect of Nitrogen sources on the growth of selected L-asparaginase producers

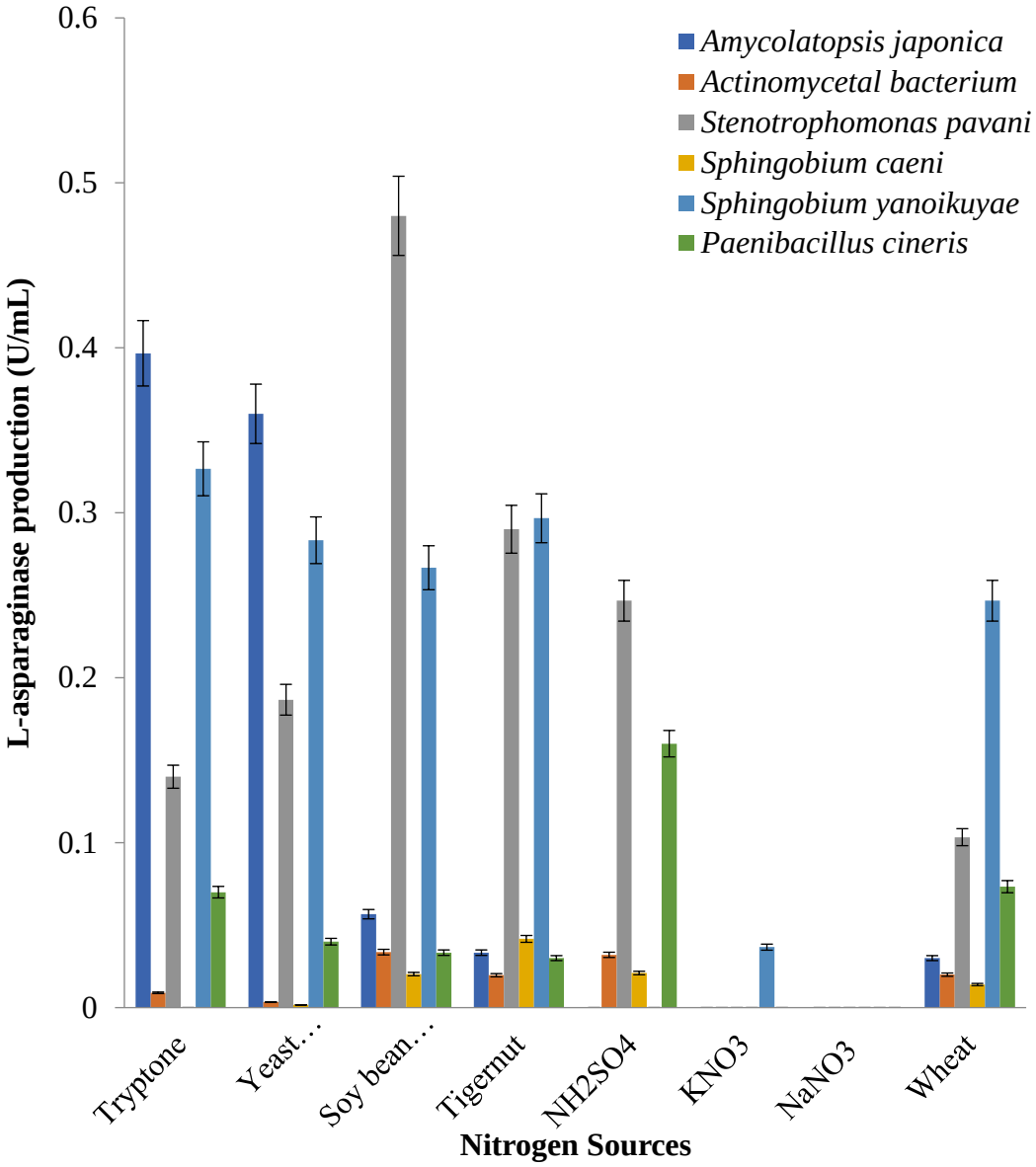


Figure 4.15: Effect of Nitrogen sources on L-asparaginase production from selected L-asparaginase producers.

When different concentrations of the substrate (L-asparagine) was used to check the effect of different substrate concentration on the growth and L-asparaginase production from the selected isolates, the responses are reported in Figures 4.16 and 4.17

Figure 4.16 revealed that 1% L-aspargine supported the highest growth of *Amycolatopsis japonica* while lowest growth was noticed when 0.5% L-asparagine was used. It was observed that there was a decrease in growth of *Amycolatopsis japonica* after the substrate concentration increased beyound 1%. *Actinomycetal bacterium* yielded highest growth when 0.5% L-asparagine was used and the lowest growth was seen when 1.5% L-asparagine was used. The maximum growth for *Stenotrophomonas pavani* was in the presence of 0.25% L-asparagine and the least growth was in the presence of 2.0% L-asparagine. The optimum growth of *Shigobacterium caenis* was noticed when 1.0% L-asparagine was used and the lowest growth was when 2.0% L-asparagine was used. There was an increase in the growth of *Shigobacterium caenis* as the subtrate increased up to 1.0% after which decrease in the growth of *Shigobacterium caenis* was noticed. *Sphingobium yanoikuyae* showed maximum growth when 0.5% L-asparagine was used in the culture medium and the lowest growth was noticed when 0.25% L-asparagine was used. A decrease in growth as the substrate increased was noticed from 0.5% to 2.0%. The maximum growth for the *Paenibacillus cineris* was in culture medium with 1.5% L-asparagine and the least growth was in the culture medium with 0.25% substrate. Increase in the growth of the *Paenibacillus cineris* was noticed with increase in the substrate (L-asparagine).

The optimum L-asparaginase production from *Amycolatopsis japonica* was achieved when 1.5% substrate was used in the culture medium with enzyme activity of 1.19 U/mL (Fig 4.17) and the minimum production was when 2.0% substrate was used with enzyme activity of 0.06 U/mL. It was observed that the higher the substrate, the higher the L- asparaginase produced. *Actinomycetal bacterium* produced maximally in the presence of 0.5% substrate with enzyme activity of 1.25 U/mL and lowest production was when 2.0% substrate was used with enzyme activity of 1.08 U/mL. Increase in L-asparaginase production was noticed with increase in the substrate. The optimum L-aspraginase production

from *Stenotrophomonas pavani* was recoded when 2.0% substrate was used with enzyme activity of 1.14 U/mL and the lowest activity was noticed in the medium with 0.25% substrate concentration with enzyme activity of (0.21 U/mL). There was gradual increase in L-asparaginase production as the substrate concentration increased. *Shigobacterium caenis* gave optimum L-asparaginase production when 2.0% substrate was used in the medium with the enzyme activity of 1.25 U/mL and the lowest L-asparaginase production was when 0.25% was used in the medium with enzyme activity of 0.30 U/mL. There was increase in production as the substrate concentrations increases. The 1.5% substrate concentration supported maximun L-asparaginase production from *Sphingobium yanoikuyae* with enzyme activity of 1.17 U/mL and the lowest L-asp production was achieved when 0.25% substrate was used with enzyme activity of 0.08 U/mL. Increase in the L-asp production was noticed with increase in substrate concentration from 0.5% to 1.5%. *Paenibacillus cineris* gave highest yield of L- asparaginase when 1.5% substrate was used in the culture medium with enzyme activity of (1.17 U/mL) and the lowest yield was noticed when 0.25% was used with enzyme activity of 0.01 U/mL.

Overall, 1% to 1.5% and 0.5- 1% L-asparagine supported the highest growth and L-asparaginase yield from *Amycolatopsis japonica* and *Actinomycetal bacterium respectively.* Increased growth for *Stenotrophomonas pavani* and *Sphingobacterium caeni* was recorded in the presence of 0.25 - 2.0% L-asparagine, but the commensurate L-asparaginase produced was when 1 – 2.0% substrate was used. *Sphingobium yanoikuyae* recorded increased growth in the presence of 0 - 5 to 1.0% L-asparagine but maximum production was when 1 – 2% L-asparagine was used. Increased growth and L-asparaginase produced was recorded for *Paenibacillus cineris* in the presence of 1.5% L-asparagine.

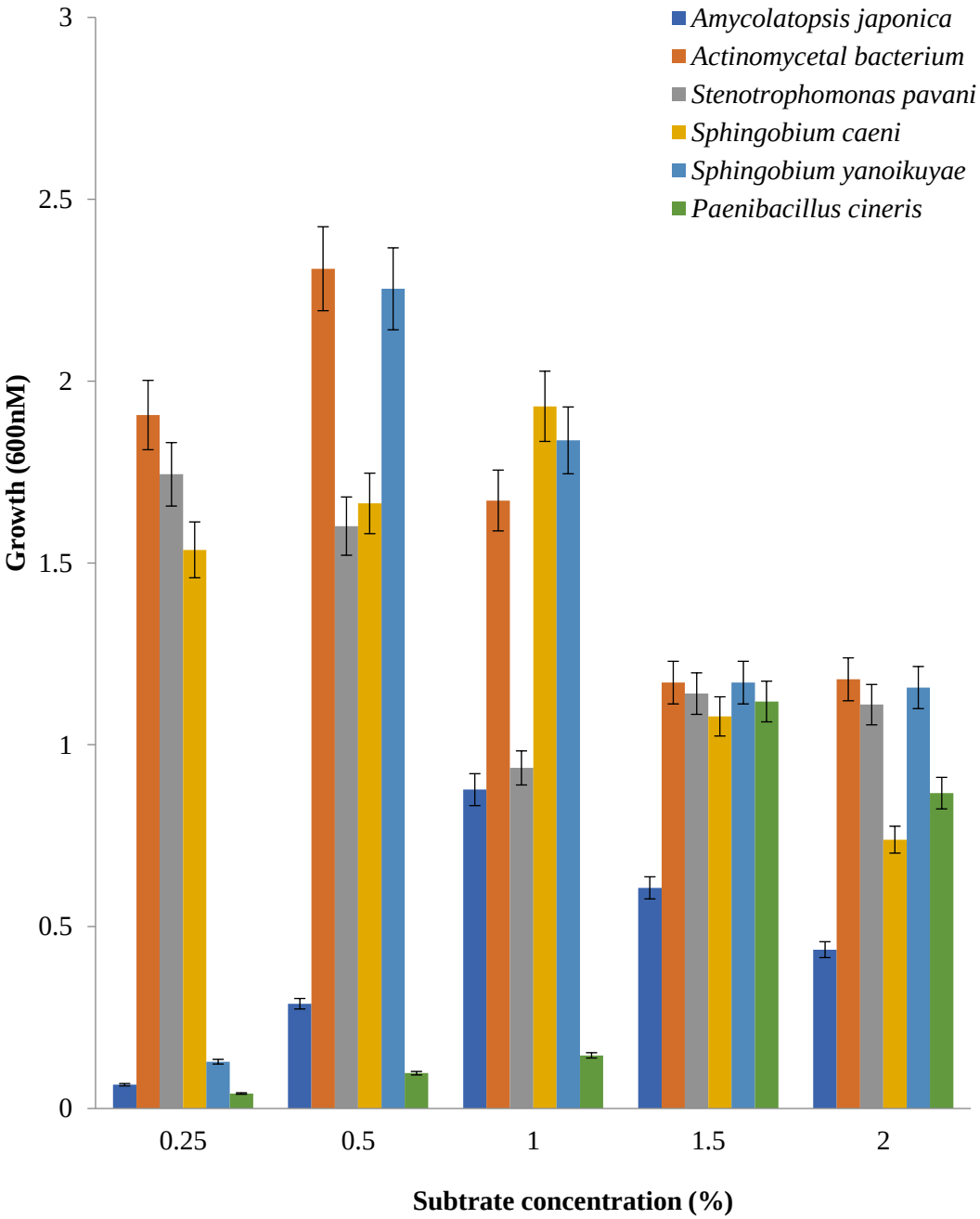


Figure 4.16: Effect of substrate concentrations on the growth of selected L-asparaginase producers

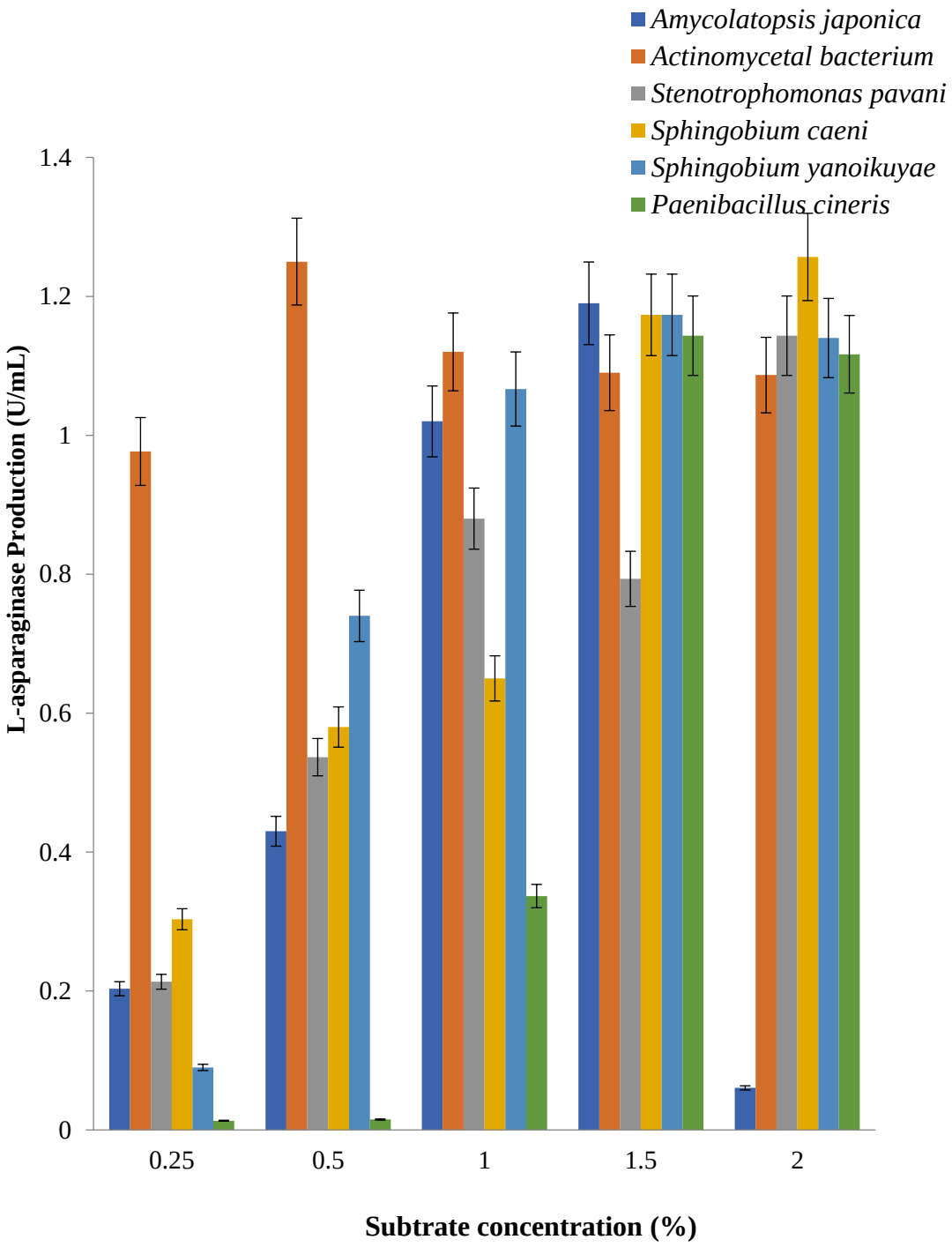

Amycolatopsis japonica
Actinomycetal bacterium
Stenotrophomonas pavani
Sphingobium caeni
Sphingobium yanoikuyae
Paenibacillus cineris
L-asparaginase Production (U/mL)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.25
0.5
1
1.5
2
Subtrate concentration (%)

Figure 4.18 illustrated the effects of metal ions on the growth of the selected isolates. It was observed that magnesium ion, followed by potassium ion were the best growth supporters of *Amycolatopsis japonica* and its least growth supporter was Zinc ion. Pottasium ion, magnesium ion and barium ion showed significant growth support for *Actinomycetal bacterium* and mercury ion less supported it growth. Barium ion, Ammonium ion and manganese ion showed highest growth support for *Stenotrophomonas pavani* and the lowest growth occurred in medium containing mercury ion. The maximum growth of *Shigobacterium caenis* was when barium ion was incorporated into the culture medium and its lowest growth was noticed when mercury ion was used, followed by Zinc ion. Magnesium ion best supported the growth of *Sphingobium yanoikuyae*, and the lowest growth of this organism was observed in the presence of mercury ion. *Paenibacillus cineris* attained its highest growth in the presence of magnesium ion and the lowest growth yield was observed in the presence of Zinc ion, Iron ion.

The L-asparaginase production pattern of the selected isolates (Figure 4.19) revealed that pottasium ion and magnesium ion best supported L-asparaginase production from *Amycolatopsis japonica* with enzyme activity of 0.48 U/mL and 0.49 U/mL. The lowest L-asparaginase yield was from barium ion with enzyme activity of 0.21 U/mL. No L- asparaginase production was achieved in the presence of manganese ion. The production of L-asparaginase from *Actinomycetal bacterium* was optimum in the presence of zinc ion and pottasium ion with the enzyme activity of 0.87 U/mL and 0.81 U/mL respectively. The minimum production was in the presence of manganese ion with enzyme activity of 0.06 U/mL. *Stenotrophomonas pavani* produced L-asparaginase maximally in the presence of barium ion followed by ammonium ion with enzyme activity of 0.93 U/mL and the lowest production was in the presence of manganese ion. No significant difference in L- asparaginase produced was recorded in the presence of pottasium ion and zinc ion (0.65 U/mL and 0.66 U/mL). Potassium ion, ammonium ion and barium ion enhanced the production of L- asparaginase from *Shigobacterium caenis* with highest enzyme activity of 1.15 U/mL, 0.79 U/mL and 0.78 U/mL respectively while the lowest L-asparaginase was in the presence of manganese ion with the enzyme of 0.08 U/mL. No L-asparaginase

production by *Shigobacterium caenis* was produced in the presence of Zinc ion. In the presence of magnesium ion, maximum L-asparaginase was produced from *Sphingobium yanoikuye* with enzyme activity of 0.52 U/mL, followed by iron ion with enzyme activity of 0.50 U/mL. The lowest L-asparaginase production was from manganese ion and Zinc ion with enzyme activity of 0.03 U/mL and 0.04 U/mL respectively. No significant difference between the L-asparaginase produced in the presence of ammonium ion and barium ion. No significant L-asparaginase was produced from *Paenibacillus cineris* in the presence of all metal tested. The highest L- asparaginase production was noticed in the presence ferric ion with enzyme activity of 0.008 U/mL and the lowest was in the presence of potassium ion with enzyme activity of 0.0003 U/mL. No L- asparaginase production was achieved in the presence of zinc ion and maganese ion.

Overall, increased growth and L-asparaginase produced for *Amycolatopsis japonica* was recorded in the presence of Mg^{2+} and K^{+}. *Actinomycetal bacterium* recorded increased growth in the presence of Mn^{2+}, K^{+} and Mg^{2+}, but recorded a commensurate L-asparaginase production in the presence of Zn^{2+} and K^{+}. *Stenotrophomonas pavani* recorded increased growth and L-asparaginase produced in the presence of Ba^{2+} and NH^{2+}. Ba^{2+}, K^{+} and NH^{2+} supported high growth and L-asparaginase production in *Sphingobacterium caeni*. *Sphingobium yanoikuyae* recorded high growth in the presence of Mg^{2+}, NH^{2+} and Ba^{2+}, but increased L-asparaginase yield was in the presence of Mg^{2+} and Fe^{2+}. *Paenibacillus cineris* recorded increased growth in the presence of Mg^{2+} and K^{+}, but the commensurate L-asparaginase produced was in the presence of Fe^{2+} and Mg^{2+}.

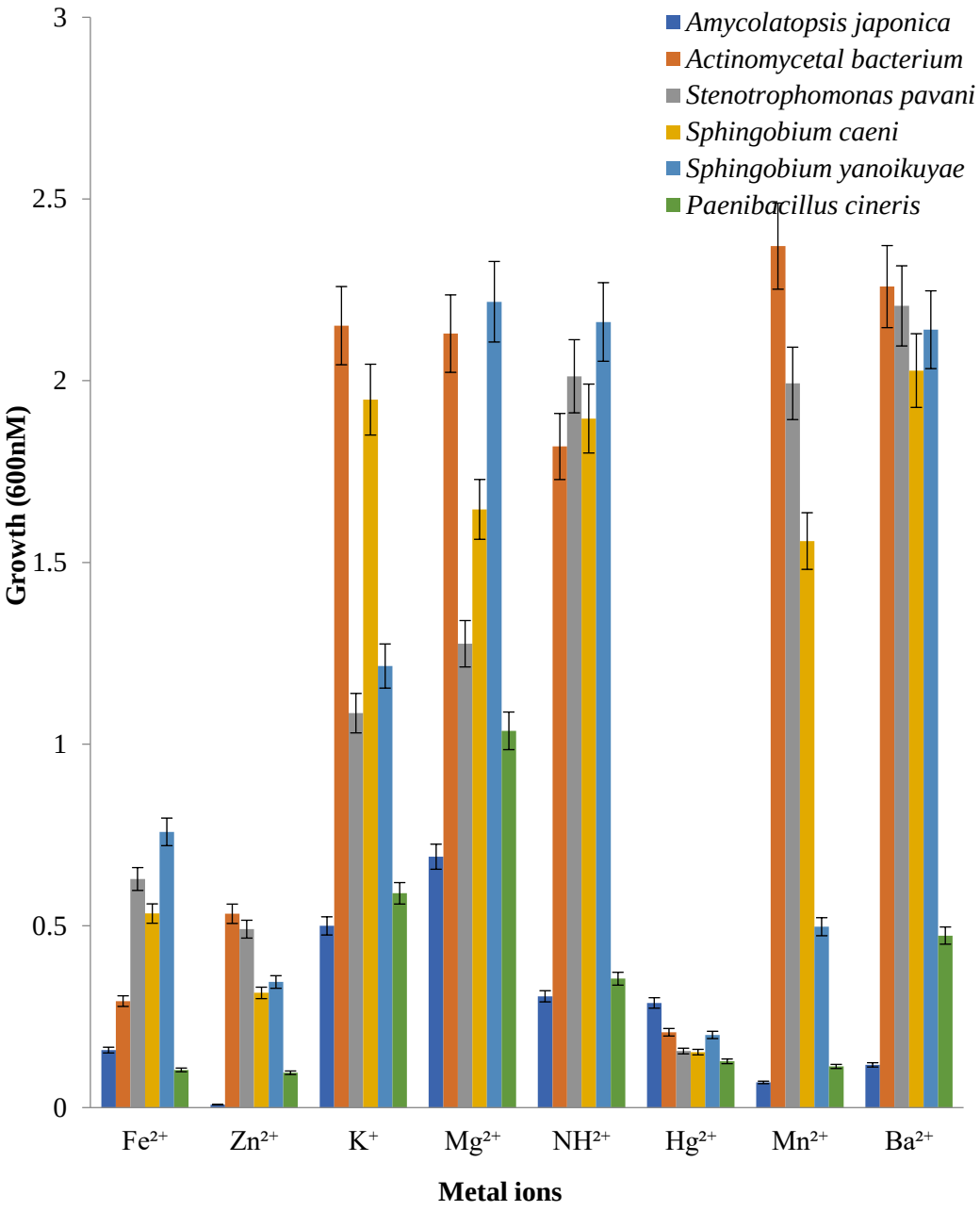


Figure 4.18: Effect of metal ions on the growth of selected L-asparaginase producers

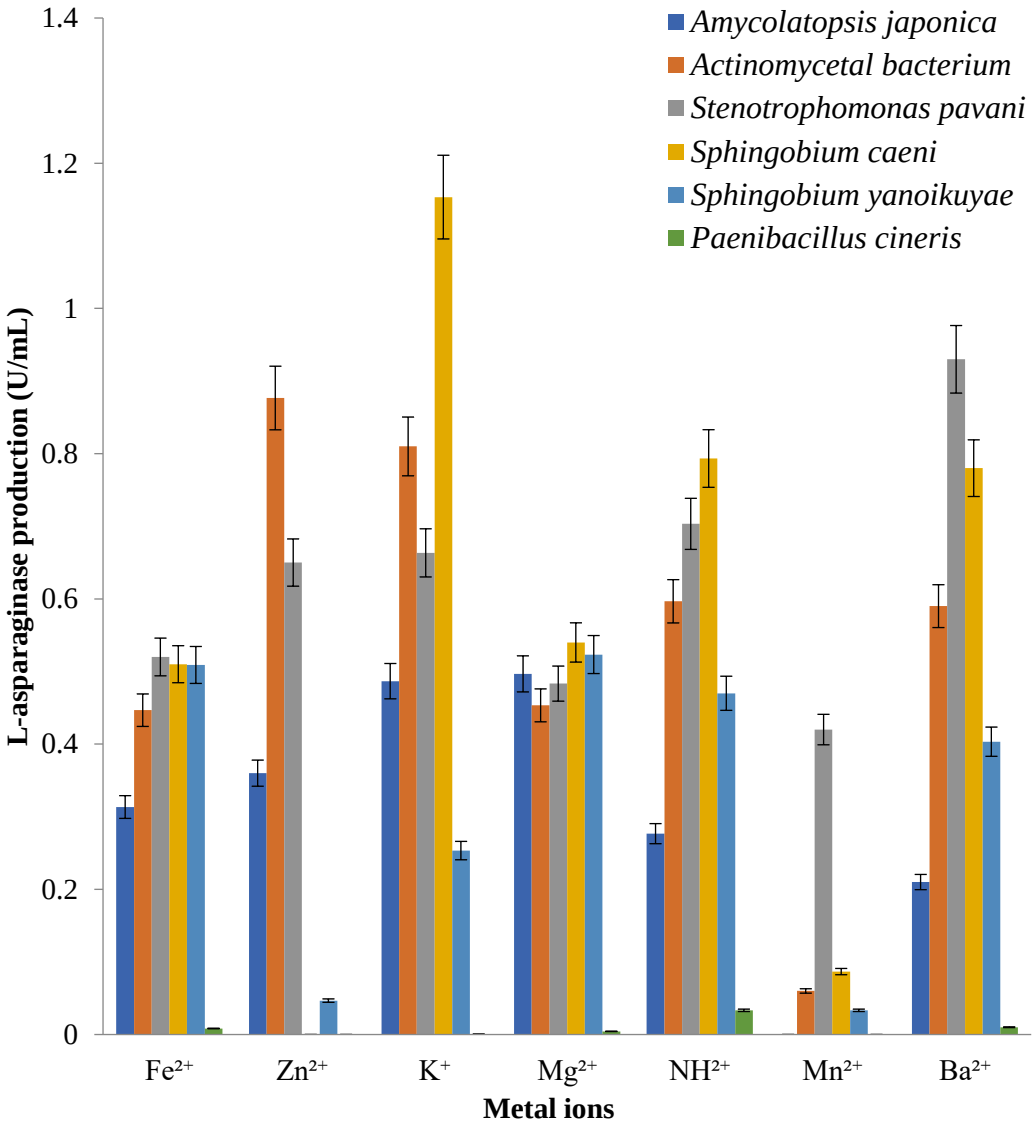


Figure 4.19: Effect of metal ions on L-asparaginase production from selected L-asparaginase producers

The influence of agitation on the growth and L- asparaginase production from selected isolates is reported in Figure 4.20 and 21. It was observed that the growth of *Amycolatopsis japonica* was enhanced with increase in speed. The highest growth was achieved at 250 rpm and the lowest growth was achieved at 100 rpm. There was decrease in the growth after the rate of 250 rpm. *Actinomycetal bacterium* achieved its highest growth) between 150 and 200 rmp and the lowest growth were achieved at the speed of 100 rmp. *Stenotrophomonas pavani* had its highest growth at the speed of 300 rpm and its lowest growth was at the speed of 100 rpm. The growth increased with increase in agitation. *Shigobacterium caenis* attained its highest growth at 250 rpm and the lowest growth was noticed at 100 rmp. The optimum growth of *Sphingobium yanoikuyae* was achieved at 200 rpm and 250 rpm. There was no significant difference in the growth at 200 and 250 rpm. The lowest growth was achieved at 100 rpm. Speed at 300 rpm favoured the growth of *Paenibacillus cineris* and the lowest growth was observed at 100 rpm. There was no significant difference between the growth of the organisms at 150 rpm and 250 rpm.

The effect of agitation on L-asparaginase production from selected isolates showed that the maximum L-asparaginase production from *Amycolatopsis japonica* was between the agitation rate of 150 rpm to 200 rpm with enzyme activity of 0.65 U/mL and 0.6 U/mL (Fig 4.21). There was no significant difference between the production at 150 rpm and 250 rpm. The lowest L-asparaginase production from this organism was at 100 rpm with enzyme activity 0.53 U/mL. *Actinomycetal bacterium* gave it optimum L-asparaginase production at 100 rpm with enzyme activity of 0.56 U/mL and the lowest production was at 150 rpm with enzyme activity of 0.33 U/mL. There was no significant difference between its production at 250 rpm and 300 rpm. The highest L-asparaginase production by *Stenotrophomonas pavani* was achieved at 150 rpm with enzyme activity of 0.43 U/mL and the lowest production was at 200 rpm with enzyme activity of 0.22 U/mL. The maximum L-asparaginase production from *Shigobacterium caenis* was observed at 250 rpm with enzyme activity 0f 0.41 U/mL and the lowest production was at 100 rpm with enzyme activity of 0.32 U/mL. The highest L-asparaginase production from *Sphingobium yanoikuyae* was observed at 150 rpm with enzyme activity of 0.49

U/mL and the lowest production was achieved at 300 rpm with enzyme activity of 0.13 U/mL. *Paenibacillus cineris* gave the highest production with enzyme activity of 0.97 U/mL at 300 rpm and the lowest production was noticed at 100 rpm with enzyme activity of 0.55 U/mL. Increase in L-asparaginase production was noticed with increased in agitation rate.

Overall, increased growth for *Amycolatopsis japonica* was recorded when cultured at agitation speed between 250 – 300 rpm, but the commensurate enzyme produced was at agitation speed between 150 – 200 rpm. *Actinomycetal bacterium* recorded a maximum growth at the speed between 150 – 300 rpm, but recorded a commensurate L-asparaginase production at the speed of 100 rpm. *Stenotrophomonas pavani* recorded increased growth at the speed between 150 – 300 rpm, but recorded maximum L-asparaginase production at the speed between 100 – 300 rpm. Agitation speed of 150 – 250 rpm supported maximum growth and L-asparaginase produced in *Sphingobacterium caeni. Sphingobium yanoikuyae* recorded high growth at agitation speed between 150 – 300 rpm, but increased L-asparaginase yield was only at agitation speed of 150 rpm. *Paenibacillus cineris* recorded increased growth and L-asparaginase production at agitation speed of 300 rpm.

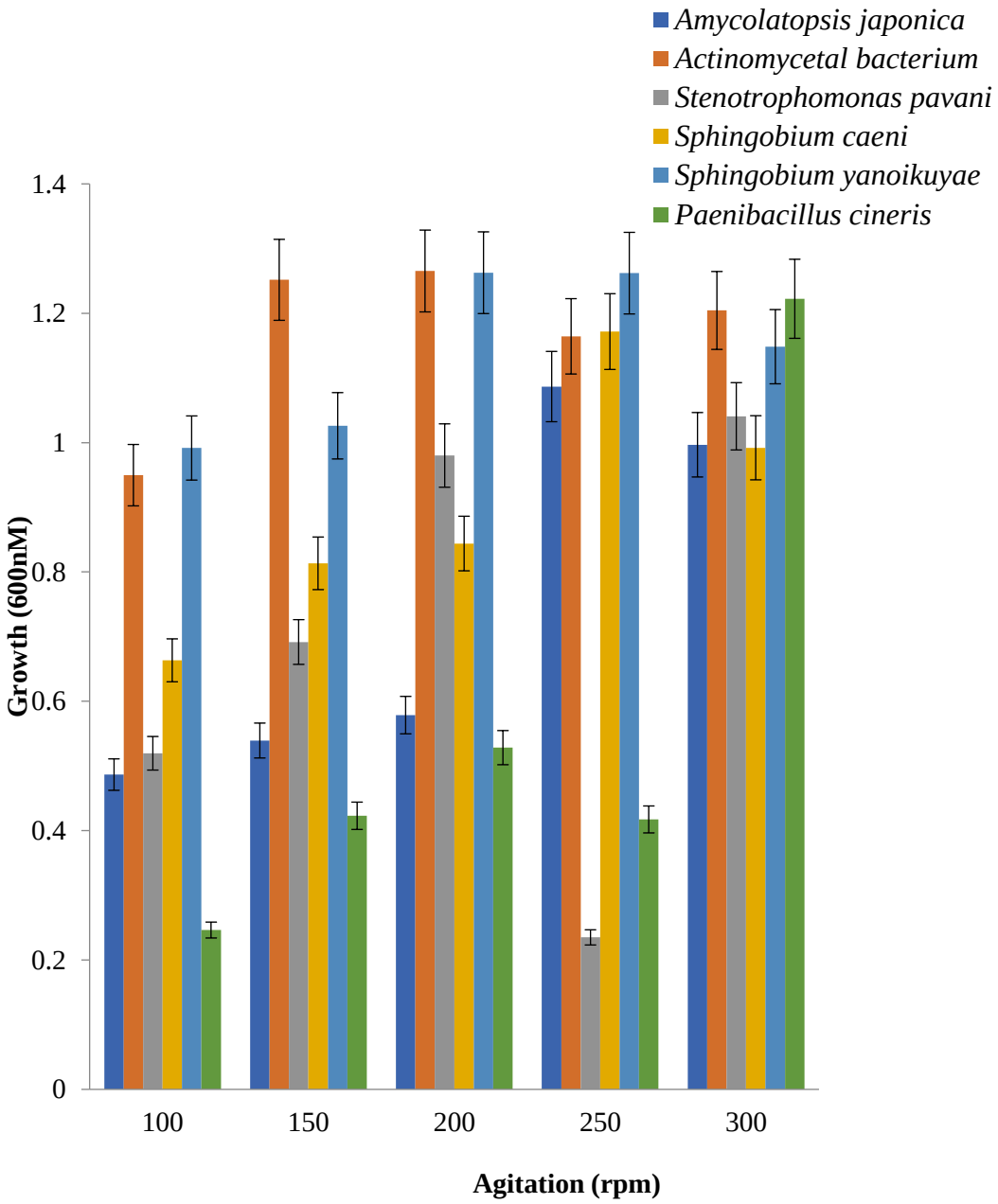


Figure 4.20: Effect of agitation on the growth of selected L-asparaginase producers

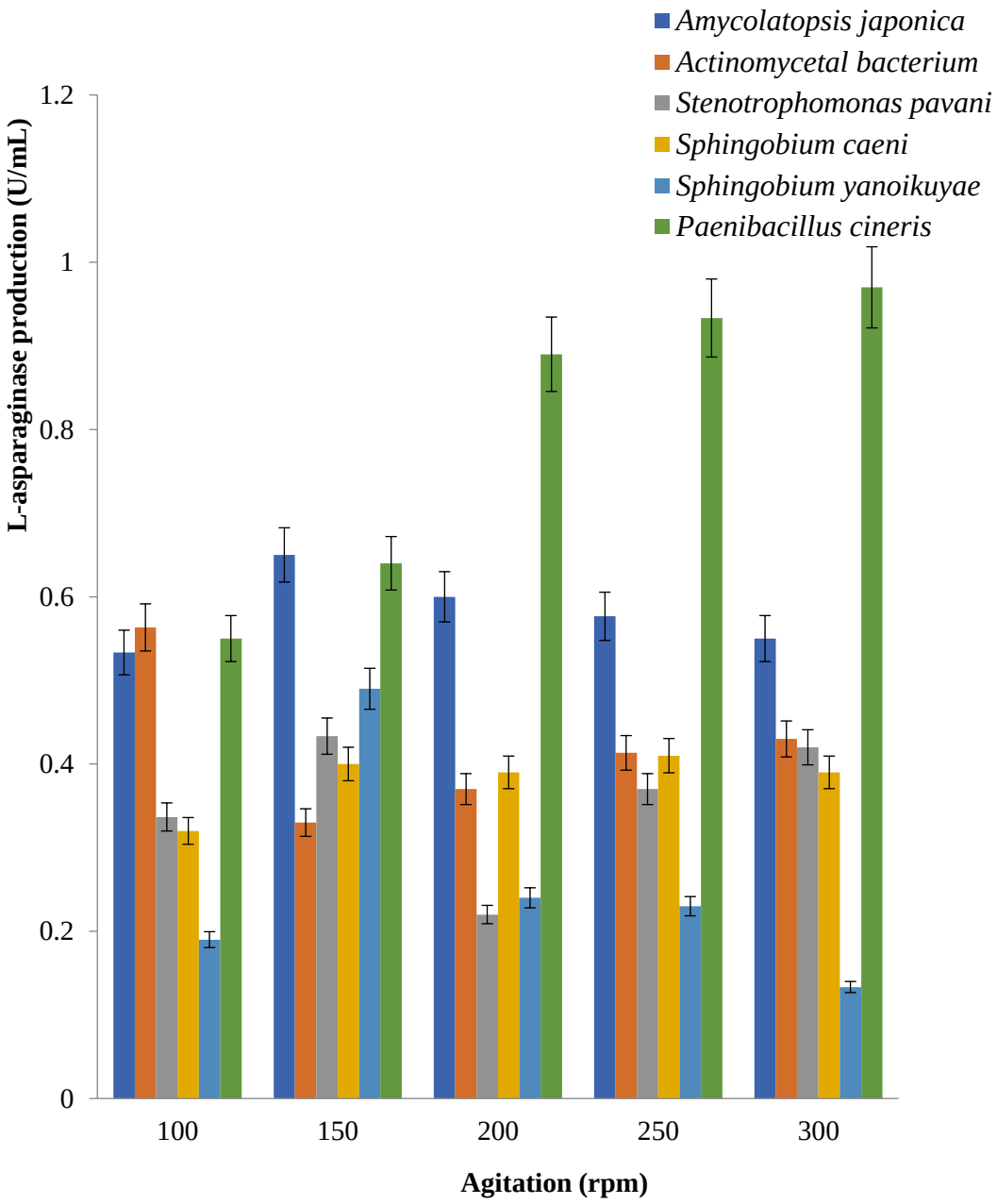


Figure 4.21: Effect of agitation on L-asparaginase production from selected L-asparaginase producers

4.5 Purification of L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuye*

4.5.1 Purification of L-asparaginase from *Amycolatopsis japonica*

The crude enzyme from *A. japonica* gave a total L-asparaginase activity of 4593.1U and total protein content of 399.47 mg with a specific activity of 11.48 U/mg. From the 3 steps purification using different concentration of ammonium sulphate, 0- 40% concentration gave a total activity of 1405.16 U, with protein content of 129.25 mg, specific activity of 10.87 U/mg, 0.94 fold purification and recovery yield of 30.6%. The treatment with 40 -80% ammonium sulphate gave 2696.41 U with 90.71 mg total protein and a specific activity of 29.7 U/mg, 2.58 purification fold and recovery yield of 58.7%. while 80- 100% ammonium sulphate treatment gave total activity of 2458.4 U, 88.36 mg protein content, specific activity of 27.82 U/mg, 2.42 purification fold with 53.5% recovery. The 40 - 80% ammonium sulphate treatment gave the highest yield of the enzyme. When different concentration of ammonium sulphate fractions were dialyzed, the dialyzed 0- 40% ammonium sulphate concentrated enzyme gave total activity of 1364.16 U with protein content of 43.05 mg, specific activity of 31.68 U/mg, 2.75 purifcation fold and 29.7% recovery. 40- 80% ammonium sulphate fractions gave total activity of 1153.6 U, 33.63 mg protein, and specific activity of of 34.28 U/mg, 2.98 purification fold and total recovery of 29.7%. Dialysed 80-100% ammonium sulphate concentrated fraction gave total activity of 1173.76 U with 18.048 protein content, specific activity of 65.03 U/mg, 5.6 fold purification and 25.55% yield recovery.

Upon further purification of each of the dialyzed fraction, the elution profile of chromatogram is shown in figure 4.28a, b and with total activity of 577.13 U protein content of 13.45 mg, specific activity of 42.90 U/mg, 3.73 purification fold and recovery yield of 12.5% for 0 – 40% ammonium sulphate fraction. 40- 80% ammonium sulphate fraction gave total activity of 656.32 U, 26.69 mg protein content specific activity of 24.58 U/mg, 2.1 fold purification and recovery yield of 14.28%. Total activity of 148.84 U, 18.42 mg protein content, specific activity of of 8.079 U/mg, 0.70 fold purification and 3.24% recovery yield was achieved with 80 - 100% ammonium sulphate fraction. All fractions which constituted a single peak and showed good L-asparaginase activity were pooled together and lyophilized (Table 4.8)

4.5.2. Purification of L-asparaginase from *Sphingobium yanoikuye*

The crude enzyme from *Sphingobium yanoikuye* gave a total L-asparaginase activity of 4774.56 U and total protein content of 5792.28 mg with a specific activity of 0.82IU/mg. From the 3 steps purification using different concentration of ammonium sulphate, 0- 40% concentration gave total activity of 609.84 U, with protein content of 540.97 mg, specific activity of 1.12 U/mg, 1.36 fold purification and recovery yield of 12.77%. The treatment with 40 - 80% ammonium sulphate gave 1963.92 U with 342.16 mg total protein and a specific activity of 5.73 U/mg, 6.96 purification fold and recovery yield of 41.1%. The 40 - 80% ammonium sulphate treatment gave the highest yield of the enzyme. When different concentration of ammonium sulphate fractions were dialyzed, the dialyzed 0- 40% ammonium sulphate concentrated enzyme gave total activity of 159.71 U with protein content of 45.30mg, specific activity of 3.52 U/mg, 4.27 purification fold and 3.34% recovery. 40- 80% ammonium sulphate fractions gave total activity of 138.43 U, 48.69 mg protein, specific activity of of 2.84 U/mg, 3.44 purification fold and total recovery of 2.89%.

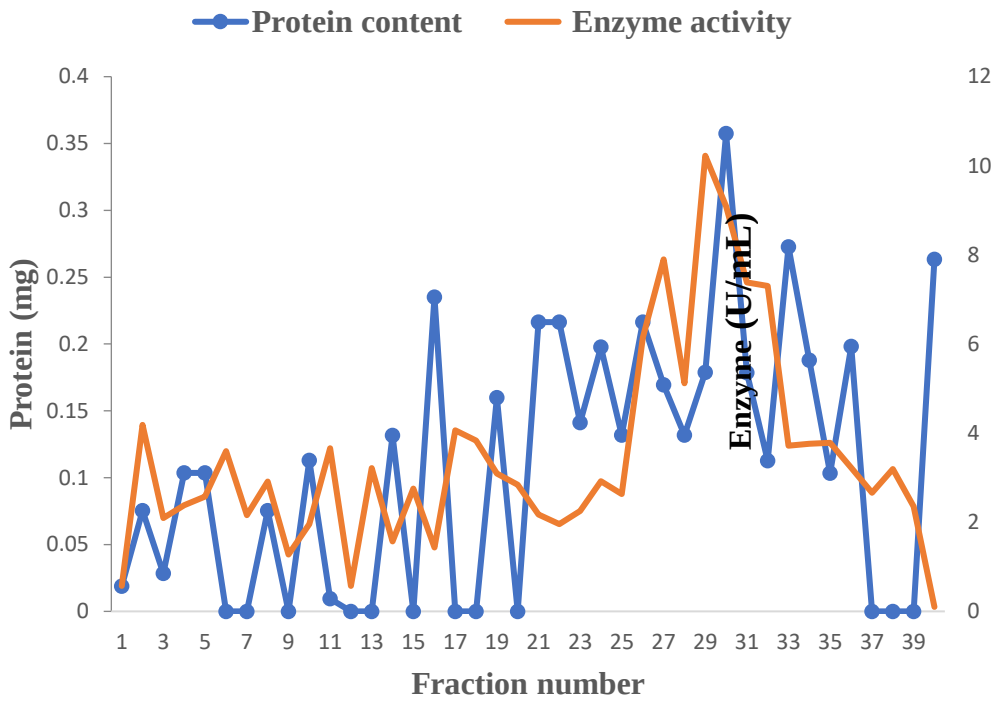
Upon further purification of the each of the dialysed fraction, the elution profile of chromatogram is shown in figure 4.29a and b with total activity of 120.17 U, protein content of 40.15 mg, specific activity of 2.99 U/mg, 3.63 purification fold and recovery yield of 2.51% for 0 – 40% ammonium sulphate fraction. 40- 80% ammonium sulphate fraction gave total activity of 127.12 U, 18.70 mg protein content, specific activity of 6.79 U/mg, 8.24 purification fold and recovery yield of 2.66%. All fractions that formed one peak and gave good enzyme activity were joined together and lyophilized (Table 4.9)

Table 4.8: Summary of the purification steps of L-asparaginase produced from Amycolatopsis *japonica*

Purification step		Total activity (U)	Total protein (mg)	Specific U/mg	Purification factor	% yield
Crude extract		4593.12	399.97	11.48366	1	100
Ammonium sulfate fractions	0- 40%	1405.6	129.25	10.87505	5.682011	30.60229
	40- 80%	2696.41	90.71	29.7255	2.588	58.70519
	80 - 100%	2458.4	88.36	27.82254	14.53876	53.52353
Dialysis	0- 40%	1364.16	43.052	31.68633	16.5552	29.70007
	40- 80%	2307.2	33.652	68.56	5.98	50.20
	80- 100%	1173.76	18.048	65.03546	33.97982	25.55474
Sephadex G- 50	0- 40%	577.136	13.4514	42.90527	22.41721	12.56523
	40- 80%	1968.96	26.696	73.75	38.53555	42.86
	80- 100%	102.48	18.424	5.5623	29.0624	22.31163

Table 4.9: Summary of the purification steps of the L-asparaginase produced by *Sphingobium yssanoikuye*

Purification step		Total activity (U)	Total protein (mg)	Specific U/Mg	Purification factor	% yield
Crude extract		4774.56	5792.28	0.824297	1	100
Ammonium sulfate fractions	0- 40%	609.84	540.97	1.127308	1.367599	12.7727
	40- 80%	1963.92	342.16	5.739771	6.96323	41.133
Dialysis	0- 40%	159.712	45.308	8.812572	10.69101	8.362655
	40- 80%	138.432	48.692	7.107533	8.622537	7.248417
Sephadex G-50	0- 40%	120.176	40.1568	14.96334	18.15285	2.517007
	40- 80%	127.12	18.706	33.9784	41.22106	2.662444



sssssFigure 4.21a: Enzyme activity and protein content elution profile of the chromatography separation of L- asparaginase from *Amycolatopsis japonica* on G-50 Sephadex column (0-40% ammonium sulphate dialysed fraction)

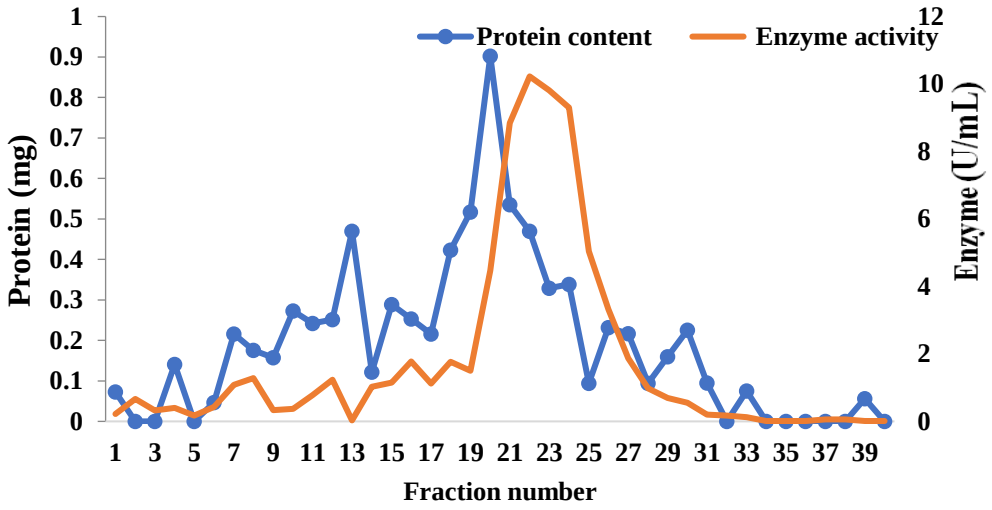


Figure 4.21b: Enzyme activity and protein content elution profile of the chromatography separation of L- asparaginase from *Amycolatopsis japonica* on G-50 Sephadex column (40-80% ammonium sulphate dialyzed fraction)

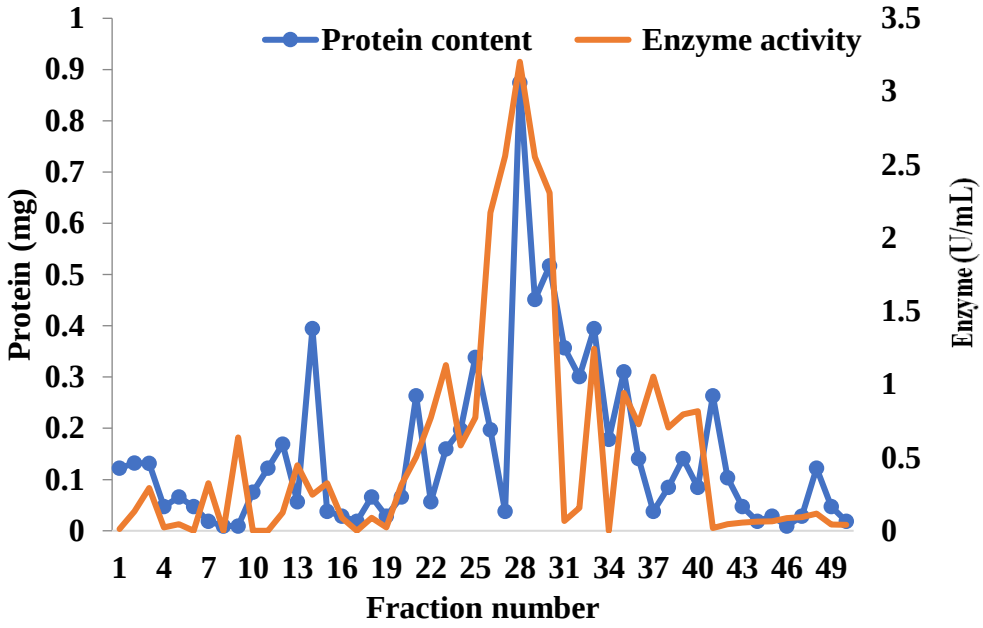


Figure 4.21c: Enzyme activity and protein content elution profile of the chromatography separation of L- asparaginase from *Amycolatopsis japonica* on G-50 Sephadex column (80-100% ammonium sulphate dialyzed fraction)

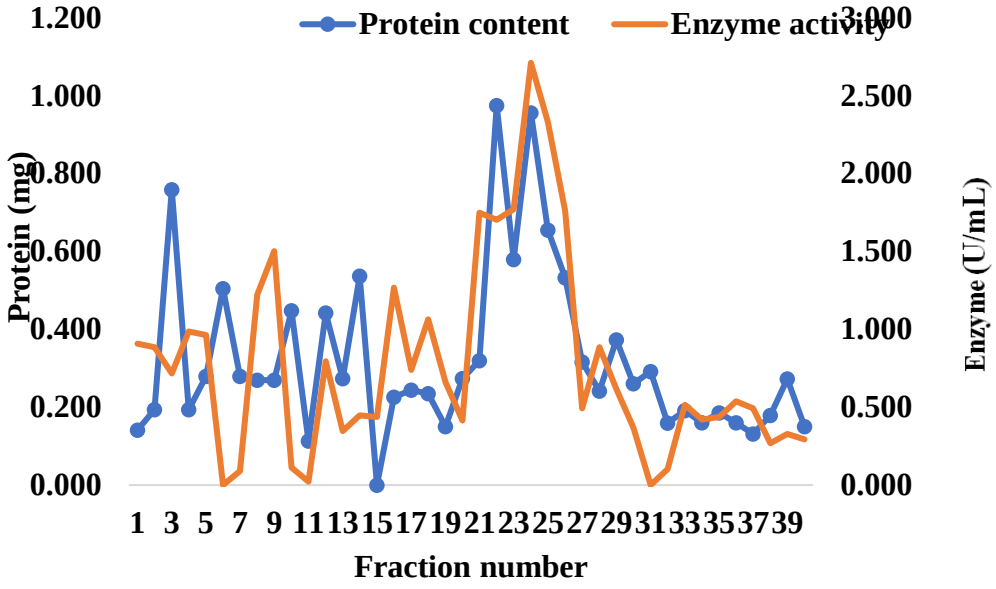


Figure 4.22a: Enzyme activity and protein content elution profile of the chromatography separation of L- asparaginase from *Sphingobium yanoikuye* on G-50 Sephaex column (0-40%) ammonium sulphate dialyzed fraction)

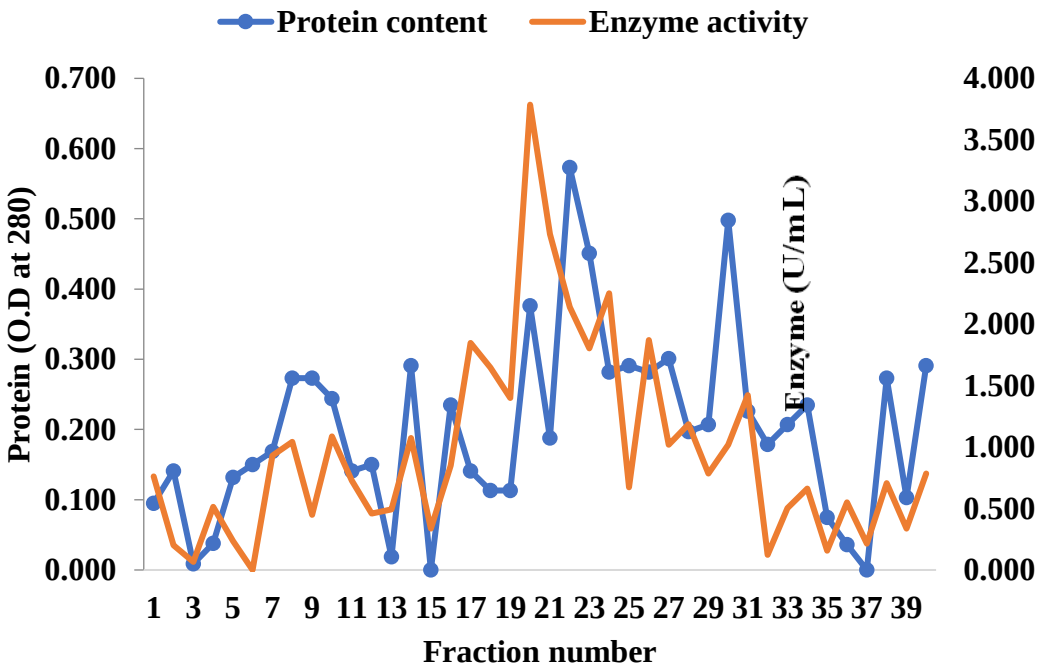


Figure 4.22b: Enzyme activity and protein content elution profile of the chromatography separation of L- asparaginase from *Sphingobium yanoikuye* on G-50 Sephaex column (40-80%) ammonium sulphate dialyzed fraction)

4.6. Characterization of L- asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae*

4.6.1. Molecular weight of L–asparaginase from *Amycolatopsis japonica*

The molecular weight of the partially purified L- asparaginese from *Amycolatopsis japonica* was 37.5 KDa (Fig. 23). Before purification, crude enzyme contain multiple band ranged from 17 KDa to 100 KDa, but after purification with G-50 gel chromatography, the pooled fraction gave a single band showing molecular weight of 37.5 KDa.

4.6.2. Molecular weight of L – asparaginase from *Sphingobium yanoikuyae*

Multiple (6) protein bands were observed from the L-asparaginase produced by *Sphingobium yanoikuyae* and the molecular weight ranges from 45 KDa to 160 KDa. But after purification with G-50 gel chromatography, the pooled fraction of the partially purified L-asparaginase gave a single band showing molecular weight of 65 KDa (Figure 4.24).

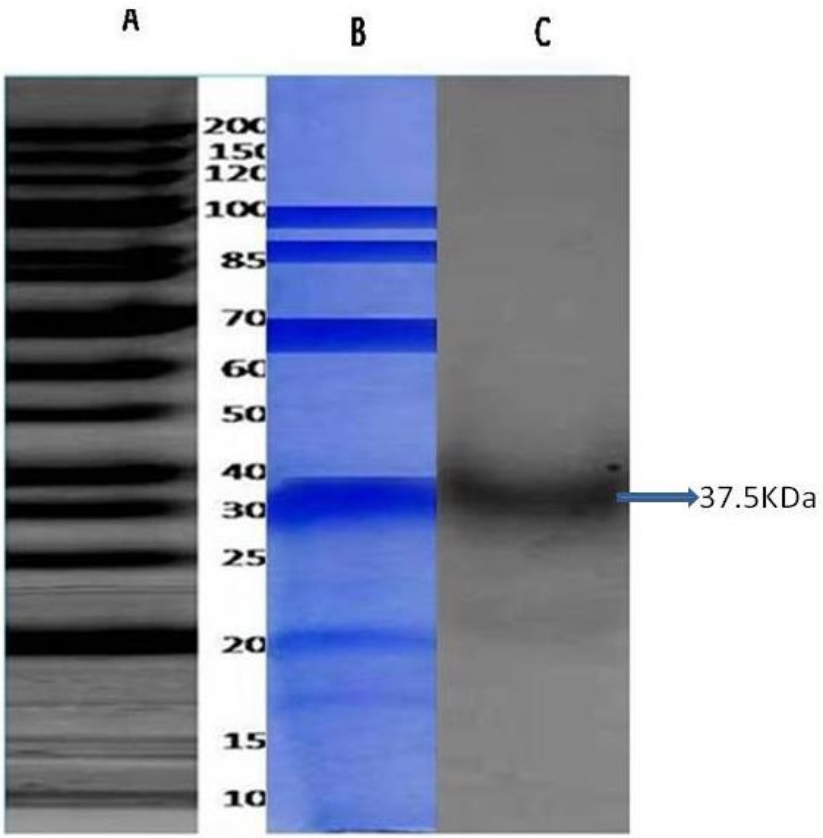


Plate 4. 2: SDS-page of L-asparaginase produced from *Amycolatopsis japonica.* Lane A: Protein marker; Lane B: crude enzyme; Lane C: Purified Enzyme

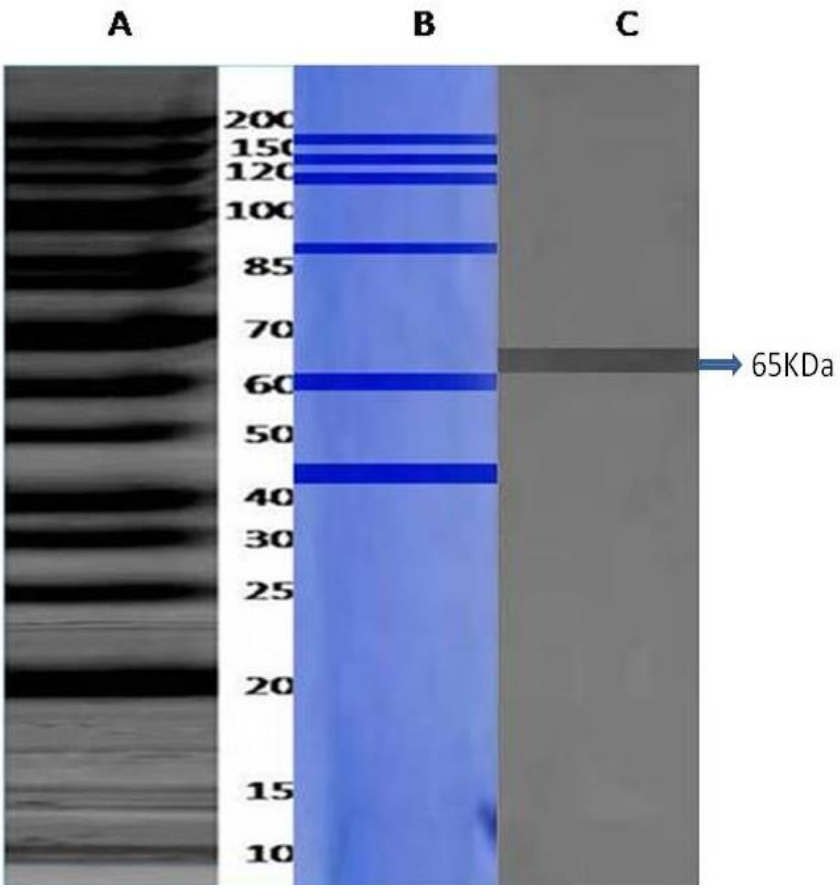


Plate 4.3: SDS-page of L-asparaginase produced from *Sphingobium yanoikuyae.* Lane A: Protein marker; Lane B: crude enzyme; Lane C: Purified Enzyme

4.6.3. Effect of temperature on activity and stability of crude and purified L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae*

The activity of crude and purified L–asparaginase from *Amycolatopsis japonica* at different temperatures (25 – 75^{o}C) is shown in (Fig 4.23) The optimum temperature for the crude enzyme was observed at 55^{o}C with 3.28 U/mL and minimum enzyme activity of 0.49 U/mL at 25^{o}C. The purified enzyme was active between 35^{o}C to 55^{o}C with optimum activity of 18.10 U/mL at 45^{o}C. The minimum activity was observed at incubating temperature of 75^{o}C with 4.99 U/mL. Increase in activity with increase in temperature was observed from 25^{o}C to 45^{o}C after which there was decrease in enzyme activity as the temperature increases.

In a similar trend, the crude enzyme from *Sphingobium yanoikuyae* showed optimum enzyme activity at 45^{o}C with 1.56 U/mL and minimum enzyme activity of 0.072 U/mL at 75^{o}C whereas, the purified enzyme from it showed optimum activity of 12.91 U/mL at 45^{o}C and the minimum activity was observed at incubating temperature of 75^{o}C with enzyme activity of 5.06 U/mL (Figure 4.24).

In terms of stability, crude L–asparaginase from *Amycolatopsis japonica* showed good stability between 25^{o}C to 45^{o}C. The activity of the enzyme was enhanced at 25^{o}C after 60 minutes of incubation. It was also observed that, the enzyme was stable within 30 minutes of incubation and retained activity of 85% at 45^{o}C. At 55^{o}C to 75^{o}C there was a gradual reduction of the enzyme activity which eventually led to loss of activity after 50 minutes of incubation (Figure 4.25). The purified L–asparaginase from *Amycolatopsis japonica* was stable at 35^{o}C and 45^{o}C, retaining up to 97% and 92% activity after 40 minutes of incubation. There was drastic loss of the enzyme activity at the incubating temperature between 65^{o}C and 75^{o}C. At 25^{o}C, L–asparaginase from *A. japonica* increased in stability up to 60% after 30 minutes of incubation (Figure 4.26).

The crude L–asparaginase from *Sphingobium yanoikuye* retained 80% of it activity at 35^{o}C after 30 minutes of incubation. At incubation temperature of 55^{o}C and 65^{o}C, the enzyme lost almost 75% of it activity after 20 minutes of incubation. After 30 minutes, 85% of the enzyme activity was retained at

incubation temperature of 75^{o}C. The enzyme retained 50% of it activity at 25^{o}C after 40 minutes of incubation (Figure 4.27). L–asparaginase from *Sphingobium yanoikuyae* retained 85% of it activity after 30 minutes of incubation at 25^{o}C and 35^{o}C. At 45^{o}C, up to 50% of the enzyme activity was retained after 60 minutes of incubation. At 55^{o}C to 75^{o}C, there was gradual decrease in the enzyme activity as the time of incubation increasing, up to 80% activity was lost after 40 minutes of incubation at this temperature (Figure 4.28).

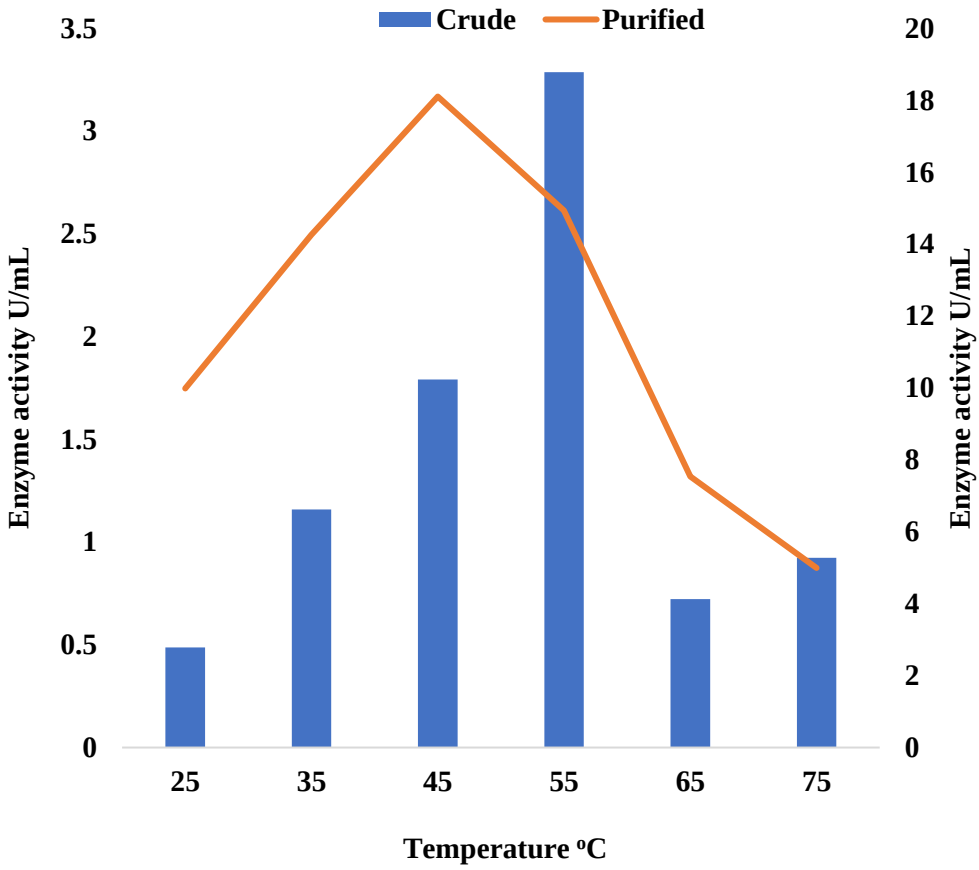


Figure 4.23: Effect of temperature on activity of crude and purified L–asparaginase produced from *Amycolatopsis japonica.*

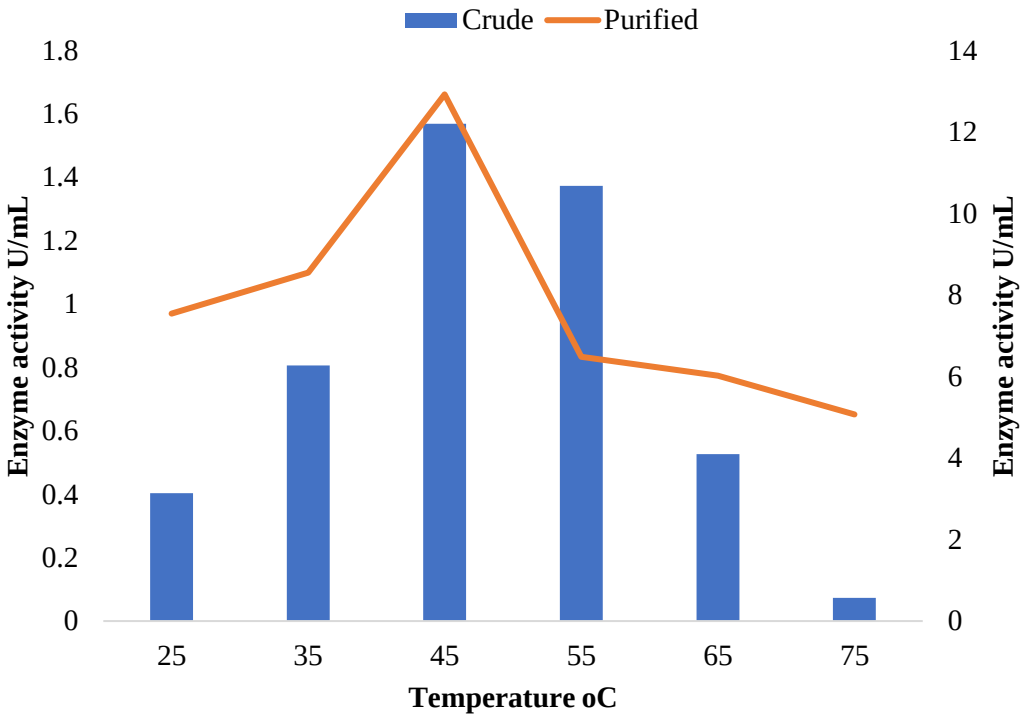


Figure 4.24: Effect of temperature on activity of crude and purified L – asparaginase produced from Sp*hingobium yanoikuyae.*

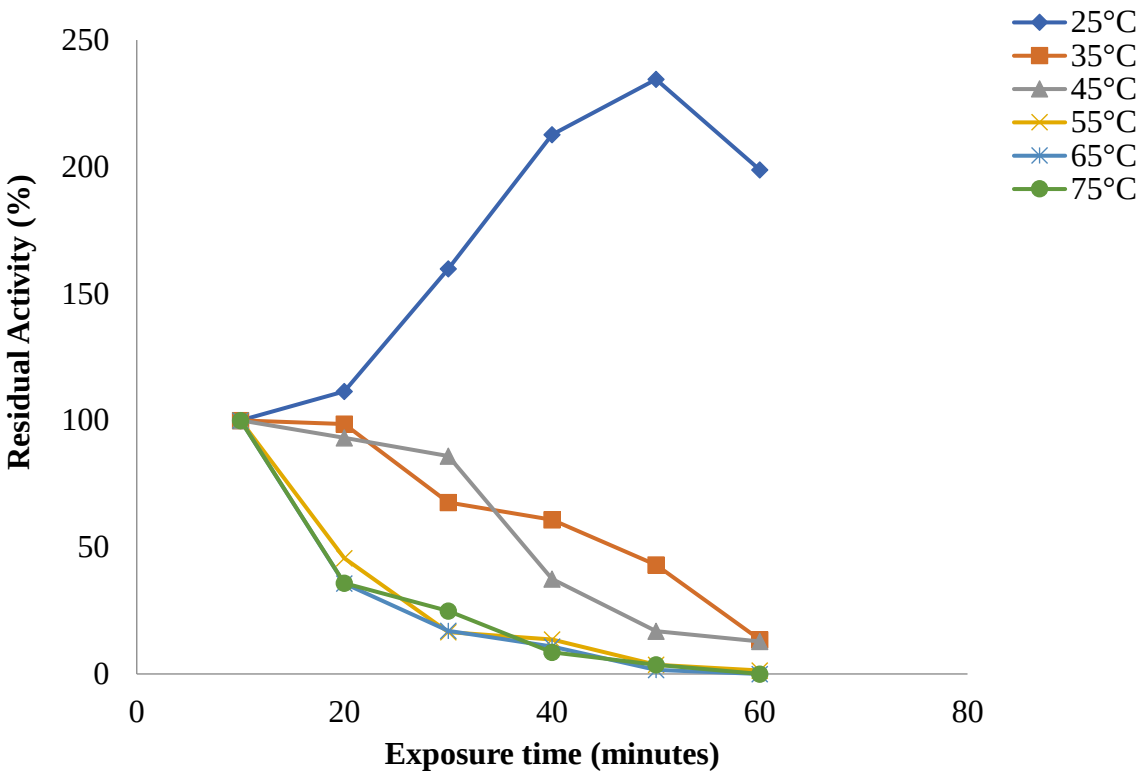


Figure 4.25: Effect of temperature on stability of crude L –asparaginase from *Amycolatopsis japonica*

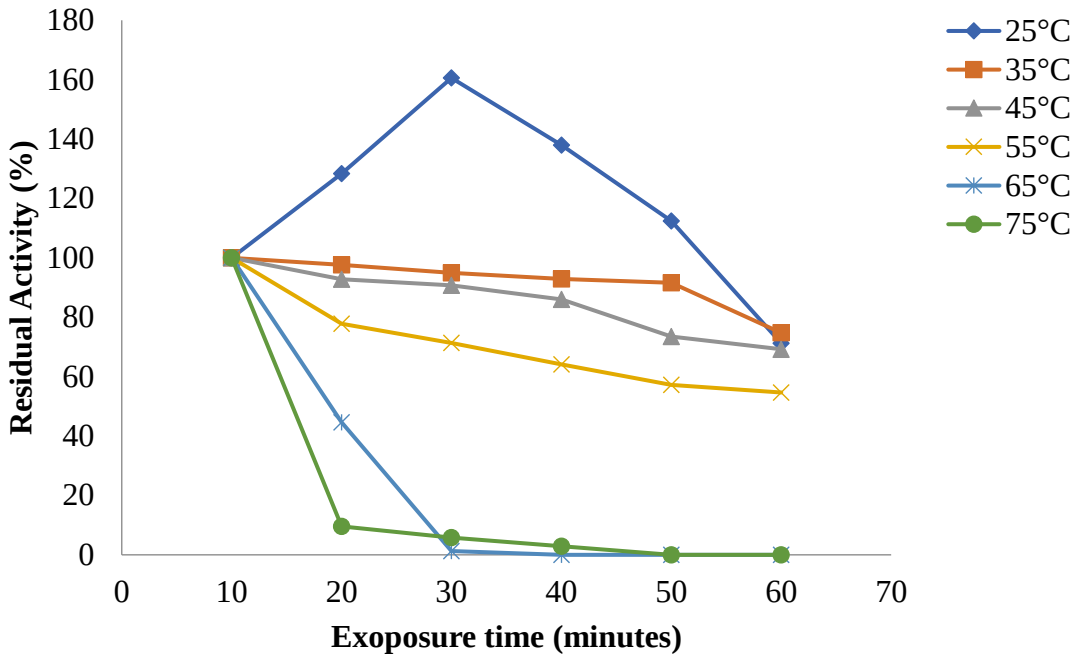


Figure 4.26: Effect of temperature on stability of purified L–asparaginase from *Amycolatopsis japonica*

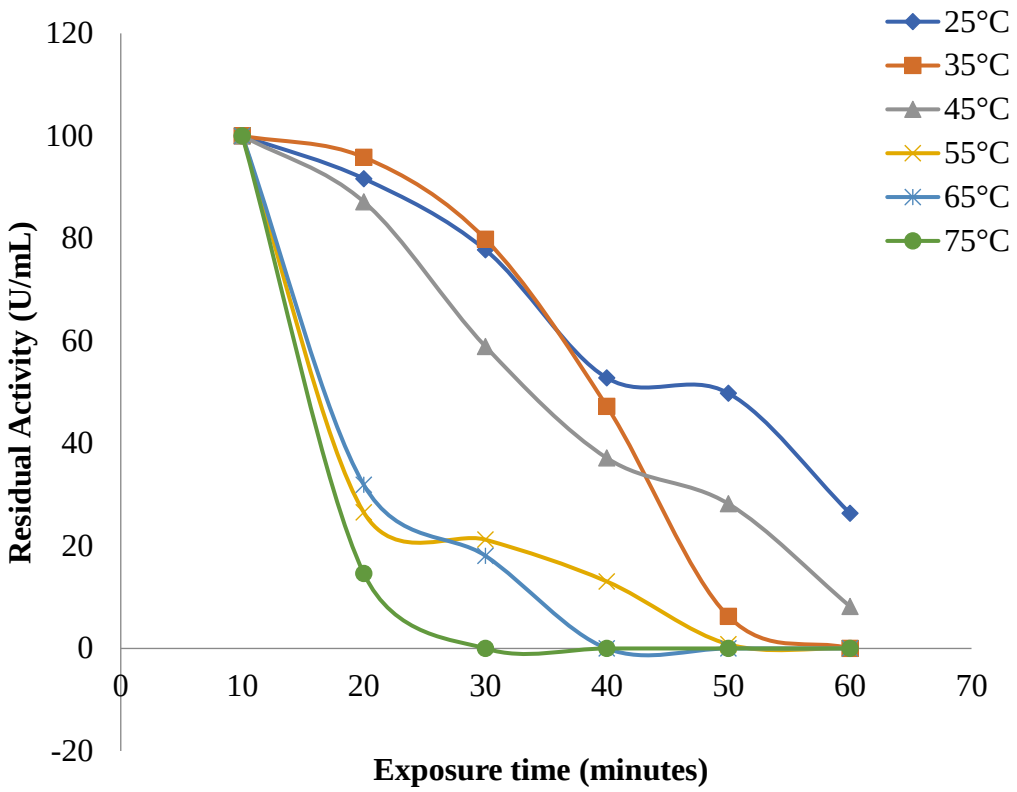


Figure 4.27: Effect of t temperature on stability of crude L–asparaginase from *Sphingobium yanoikuyae*

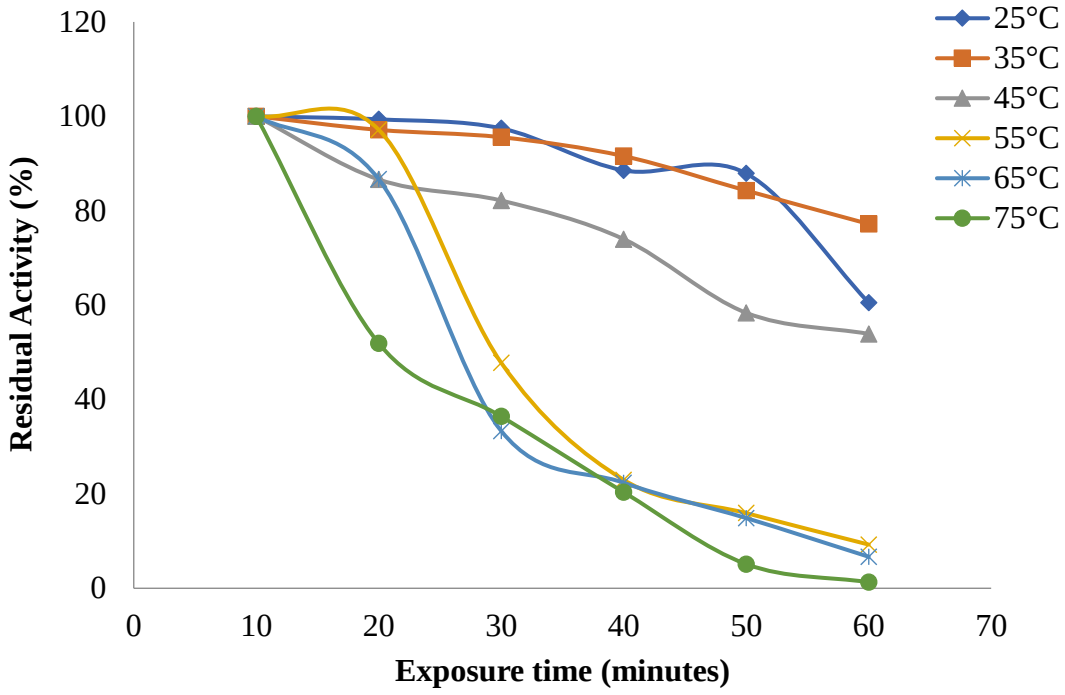


Figure 4.28: Effect of temperature on stability of purified L–asparaginase from *Sphingobium yanoikuyae*

4.6.4. Effect of pH on activity and stability of crude and purified L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae*

The effect of pH on L-asparaginase activity was studied using pH values 4.0 to 10 for both crude and purified enzyme (Fig 4.29 – Fig 4.44). From the result (as given in figure 4.29), the crude L-asparaginase from *Amycolatopsis japonica* showed higher activity between pH 6 and 9 with the highest activity of 3.167 U/mL at pH 8.0 and the lowest activity at pH 10 with the enzyme activity value of 0.1 U/mL. Also, the purified enzyme from this organism was active between pH 6 and 8 and a decrease in activity was noticed from pH 9 to 10. The maximum activity of the enzyme was also found in pH 8.0 with an increase value of 8.16 U/mL and lowest activity found at pH 10 with the value of 0.81 U/mL.

The crude enzyme from *Sphingobium yanoikuyae* showed highest activity at pH 8 (3.13 U/mL) and the lowest activity at pH10 with the enzyme activity value of 0.20 U/mL. Increase in the enzyme activity as the pH increased was recorded until pH 8 after which there was a gradual reduction. The purified enzyme also showed higher activity between pH 6 and 8 and a decrease in activity was noticed from pH 9 to 10. The maximum activity of the enzyme was found in pH 8.0 with an increase value of 2.15 U/mL and lowest activity at pH 10 with the value of 0.17 U/mL (Figure 4.30).

The crude enzyme from *Amycolatopsis japonica* showed good stability from pH 5 to 9 and nearly retained 70% of its activity after 40 minutes of incubation at pH 8 (Figure 4.31). The highest stability was found in pH 5 with 74% activity after 50 minutes of incubation. A 7% and 21% enzyme activity remained after 50 minutes incubation at pH 4 and 9 respectively, and total loss of activity was observed at pH 10 after 10 minutes of incubation at 37^{o}C. The purified enzyme from *Amycolatopsis japonica* showed good stability from pH (6-9) and nearly retained 50% of its activity after 40 minutes of incubation. At pH 4 and 5, up to 60% of the activity was lost after 30 minutes of incubation. There was a drastic loss of enzyme activity after 10 minutes of incubation at pH 5 and pH 10 (Figure 4.32).

The crude enzyme from Sp*hingobium yanoikuye* showed good stability at pH 4 and nearly remains 66% of it activity after 60 minutes of incubation. At pH5, 63% of the enzyme activity was retained after 40 minutes of incubation. The enzyme

retained 45% of it activity at pH 6 and 7, after 50 minutes of incubation. At pH 8 to 10, a drastic loss in the enzyme activity was observed and total loss of the enzyme activity was recorded after 10 minutes of incubation at pH 10 (Figure 4.33). The purified enzyme from *Sphingobium yanoikuyae* retained 77% of its activity after 40 minutes of in cubation at pH 4. At pH 8, 50% of the enyzme activity was retained after 30 minutes of incubation. A total loss of the enzyme activity was recorded after 10 minutes of incubation at pH 10 (Figure 4.34).

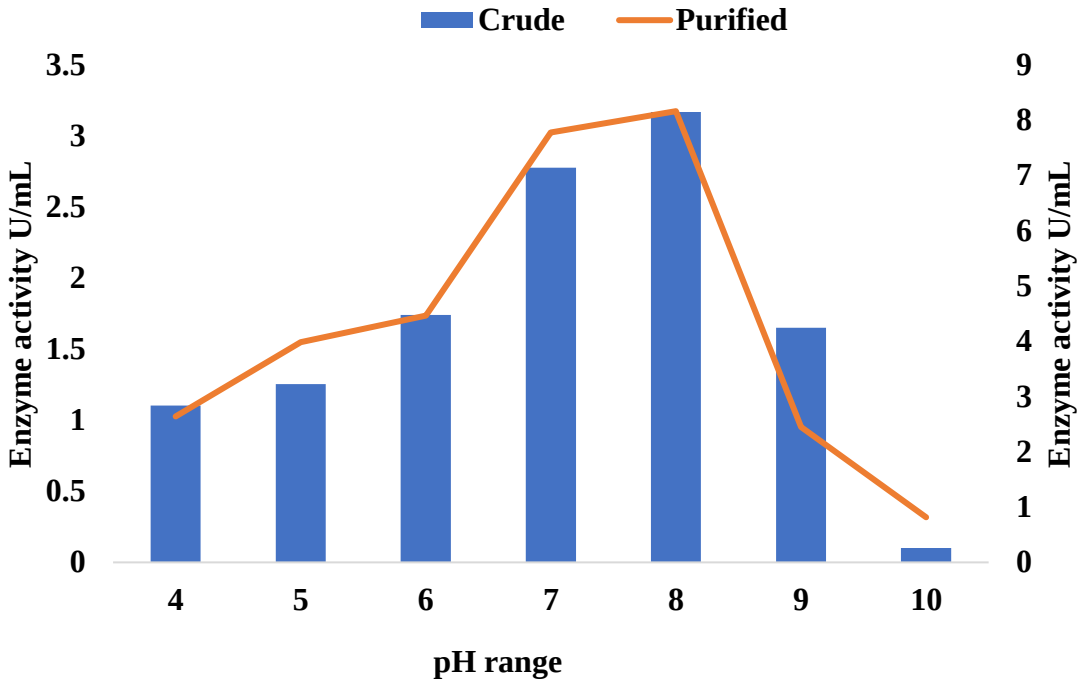
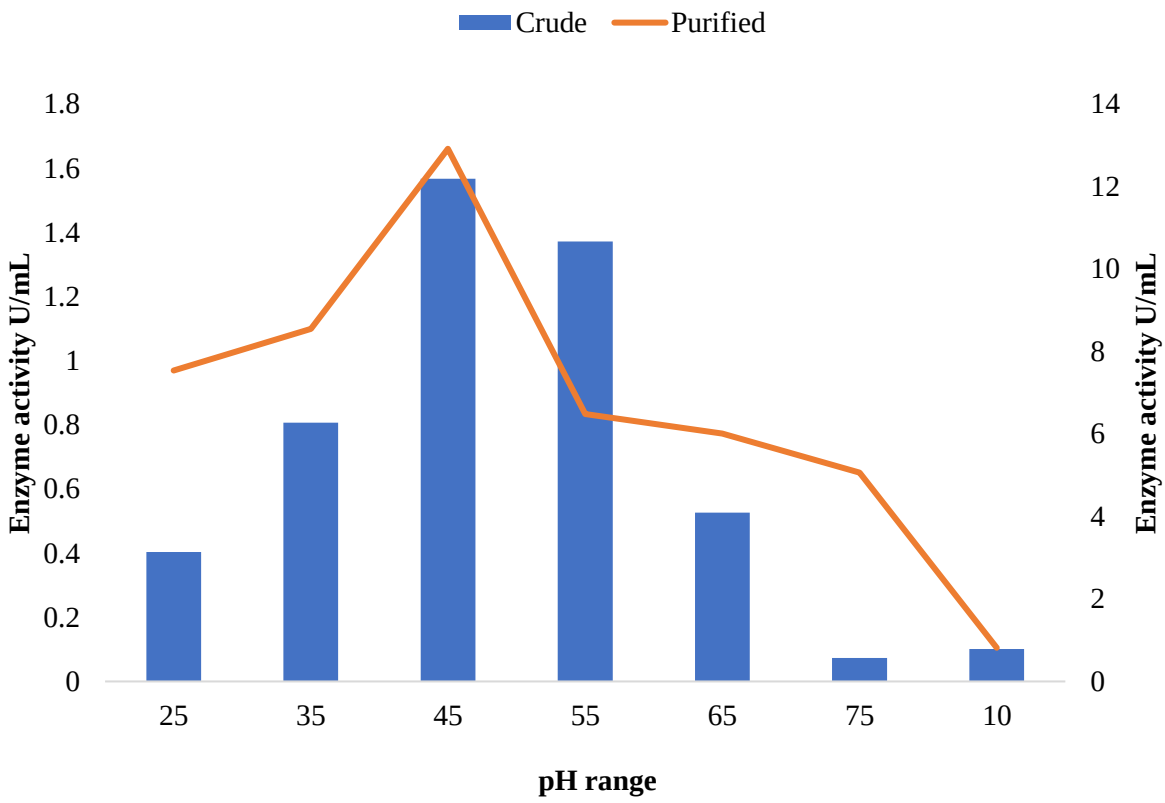


Figure 4.29: Effect of pH on activity of crude and purified L–asparaginase produced from *Amycolatopsis japonica.*



4.30: Effect of pH on activity of crude and purified L–asparaginase produced from *Sphingobium yanoikuyae.*

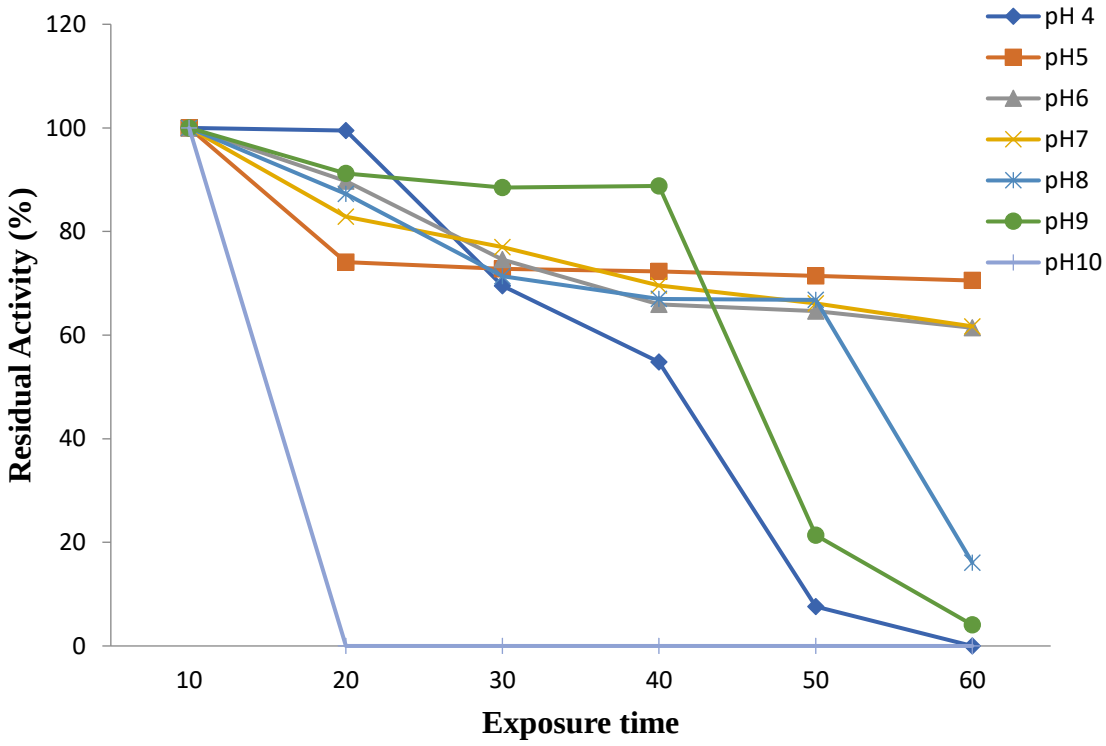


Figure 4.31: Effect of pH on stability of crude L–asparaginase from *Amycolatopsis japonica*

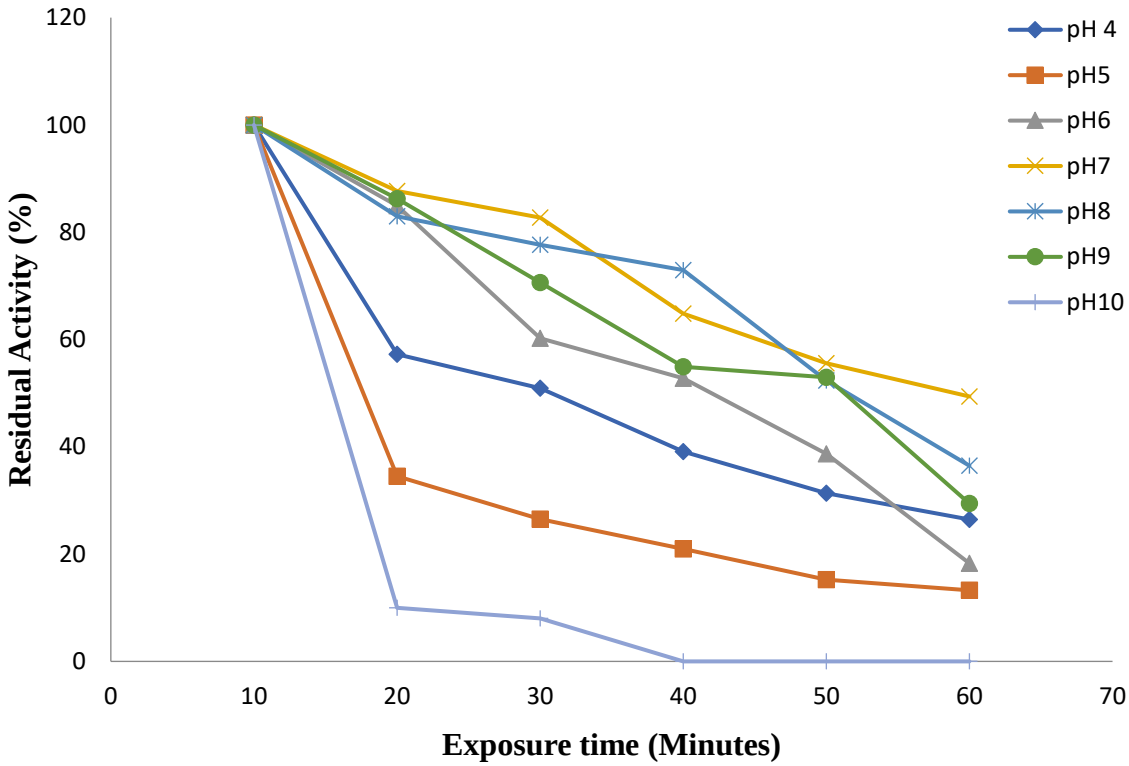


Figure 4.32: Effect of pH on stability of purified L –asparaginase from *Amycolatopsis japonica*

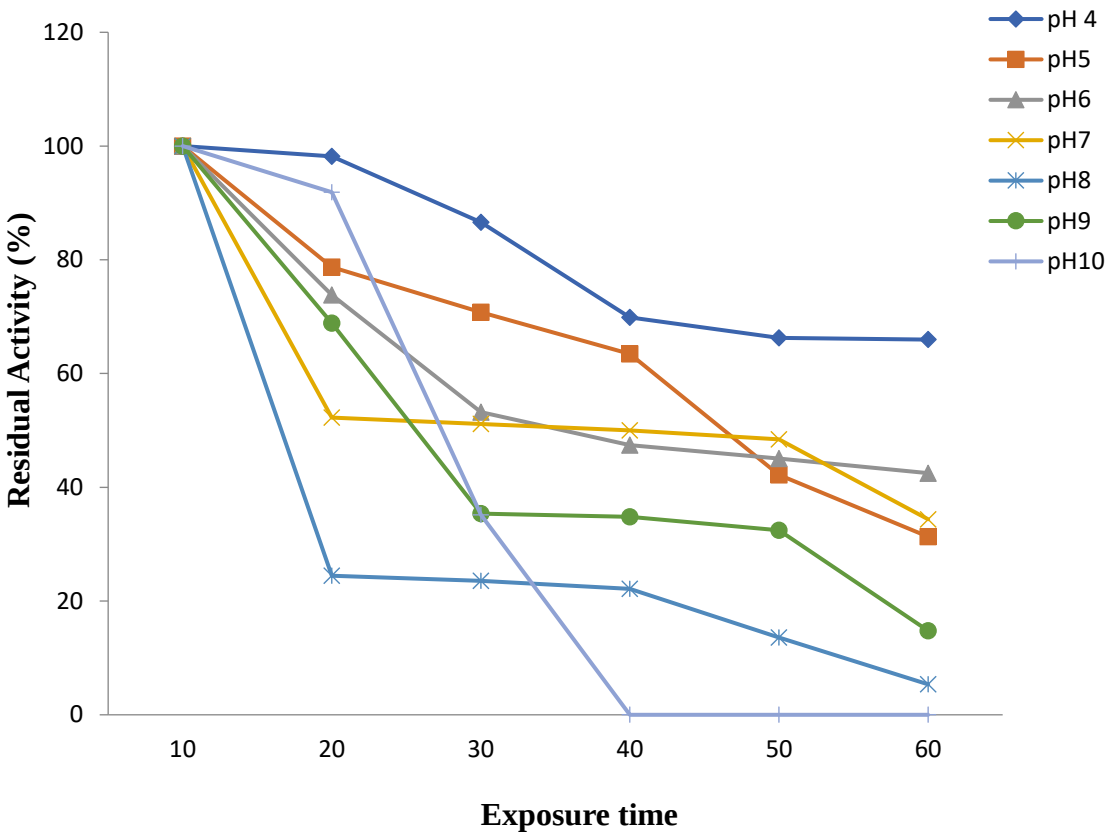


Figure 4.33: Effect of pH on stability of crude L–asparaginase from *Sphingobium yanoikuyae.*

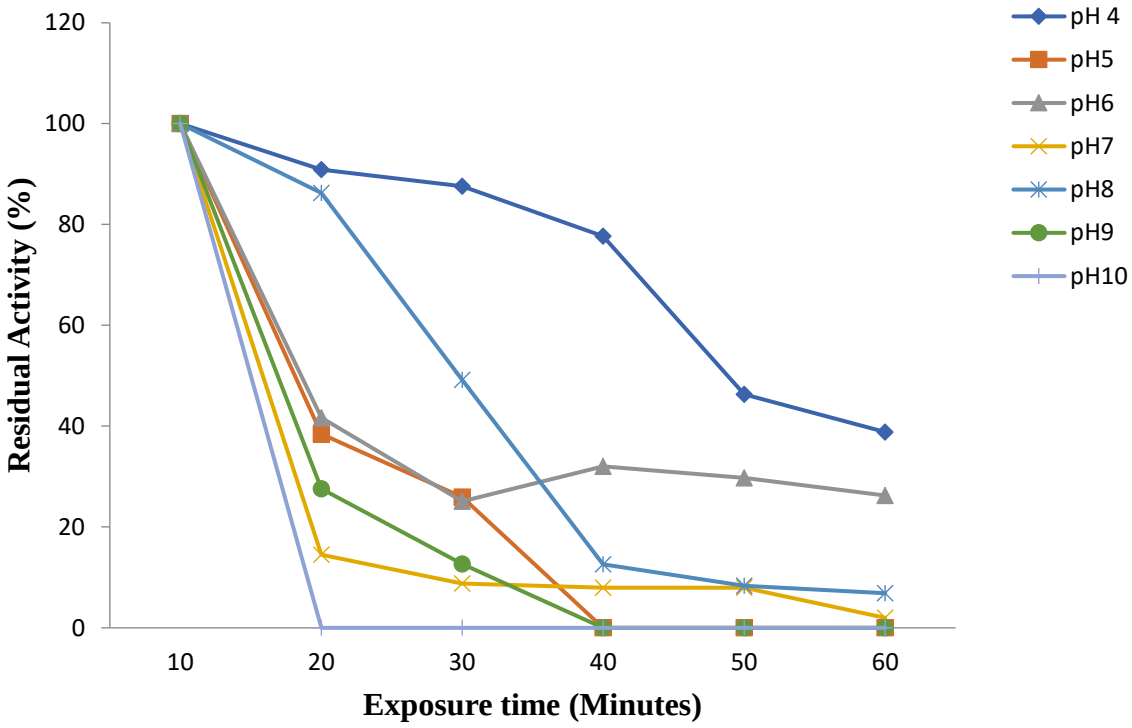


Figure 4.34; Effects of pH on stability of purified L –asparaginase from *Sphingobium yanoikuyae*

4.6.5. Effect of metal ions on activity and stability of crude and purified L-asparaginase from *Amycolatopsis japonica and Sphingobium yanoikuyae*

Fig 4.35 to 4.40 showed the effect of different metal ions on the crude and purified enzyme activity and stability of *Amycolatopsis japonica and* Sp*hingobium yanoikuyae.* From the results, Ca^{2+}, Mg^{2+} and Fe^{2+} supported crude L–asparaginase activity from *Amycolatopsis japonica* (1.98, 1.8 U /mL and 1.54 U/mL, respectively), and showed lower activity when incubated with Zn^{2+} (0.23 U/mL). The purified L-asparaginase from *Amycolatopsis japonica* showed higher enzyme activity in the presence Ca^{2+}, Fe^{2+}, K^{+} and Mg^{2+}. The maximum activity was noticed in the presence of Mg^{2+} with activity of 13.78 U/mL while lower activity was noticed in the presence of Hg^{2+} with enzyme activity value of 4.56 U/mL. The crude L-asparaginase from *Sphingobium yanoikuyae* showed higher enzyme activity in the presence Mg^{2+}, Na^{+} and Ca^{2+} (1.61 U, 0.89 and 0.72 U/mL, respectively). The maximum activity was noticed in the presence of Mg^{2+} with enzyme activity of 1.61 U/mL while lowest activity was noticed in the presence of Hg^{2+} with enzyme activity of 0.16 U/mL (Figure 4.35). Mg^{2+}, Fe^{2+}, and Zn^{2+} showed higher support for purified L–asparaginase from *Sphingobium yanoikuyae* with activity value of 7.49 U/mL, 6.14 U/mL and 5.33 U/mL respectively and lowest activity was observed when incubated with Hg^{2+} and Ca^{2+} with enzyme value of 3.16 U/mL (Figure 4.36)

The crude enzyme from *Amycolatopsis japonica* retained up to 70% of its activity after 30 minutes of incubation in the presence Na^{2+}, but lost up to 98% of its activity in the presence of Ca^{2+} after 30 minutes of incubation. Good stability was observed in the presence of Mg^{2+} as 50% of its activity was retained after 60 minutes of incubation. L-asparaginase from *Amycolatopsis japonica* showed no stability in the presence of Zn^{2+} as total loss of the activity was observed after 20 minutes of incubation (Figure 4.37). Purified L-aspraginase from *Amycolatopsis japonica* showed good stability when incubated in the presence of Mg^{2+} and K^{+} for 60 minutes; its activity was enhanced with 3% in the presence of Mg^{2+}. The stability of the enzyme reduced with increase in temperature in the presence of Fe^{2+} and Zn^{2+}. Up to 56% enzyme activity was recorded after 60 minutes of incubation at 37^{o}C in the presence of Ca^{2+}. There was drastic loss of enzyme activity in the presence of Hg^{2+} after 10 minutes of incubation (Figure 4.38).

In a similar trend, crude enzyme from *Sphingobium yanoikuyae* showed good stability when incubated in the presence of Mg^{2+} and Na^{+}, as it retained up to 60% of it activity after 60 minutes of incubation. The stability of the enzyme reduced with increase in temperature in the presence of Fe^{2+} and K^{+}. Up to 90% of the enzyme activity was noticed in the presence of Hg^{2+} after 30 minutes of incubation (Figure 4.39). The purified L-asparaginase from *Sphingobium yanoikuyae* showed good stability by retaining up to 70% of its activity after 60 minutes of incubation in the presence Mg^{2+}. In the presence of Ca^{2+}, the enzyme lost 50% of its activity after 40 minutes of incubation. The enzyme retained 90% of its activity after 40 minutes of incubation in the presence of Zn^{2+}. The enzyme retained 80% activity in the presence of Na^{+} and up to 80% loss of activity was noticed in the presence of Hg^{2+} after 40 minutes of incubation (Figure 4.40).

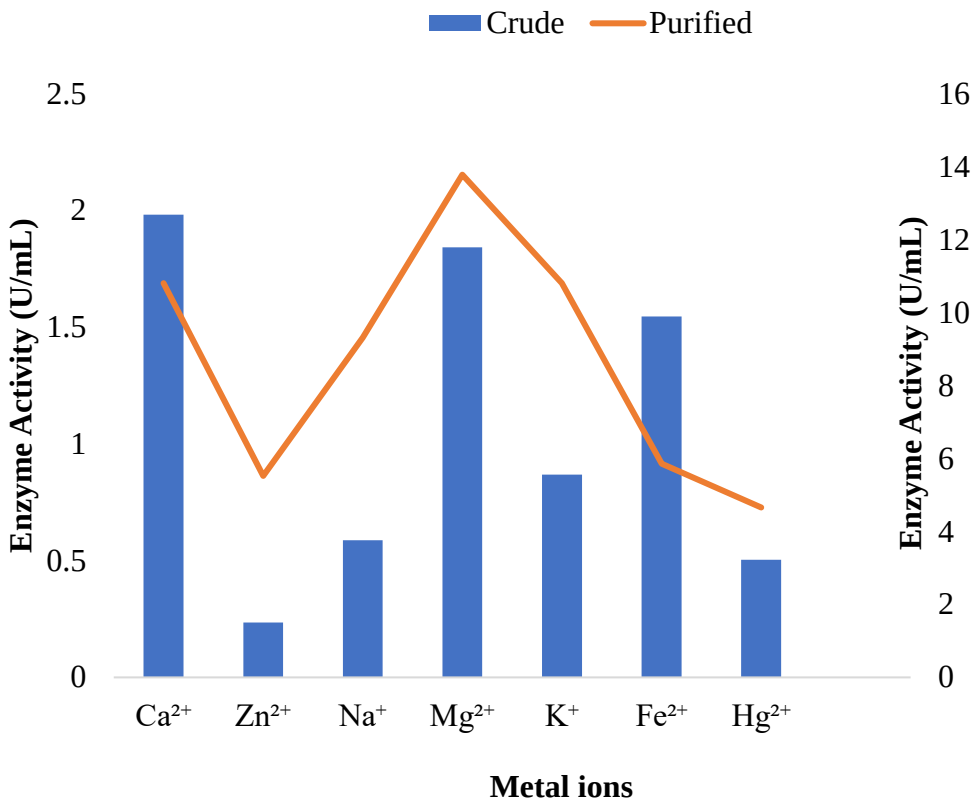


Figure 4.35: Effect of metal ions on activity of crude and purified L–asparaginase produced from *Amycolatopsis japonica.*

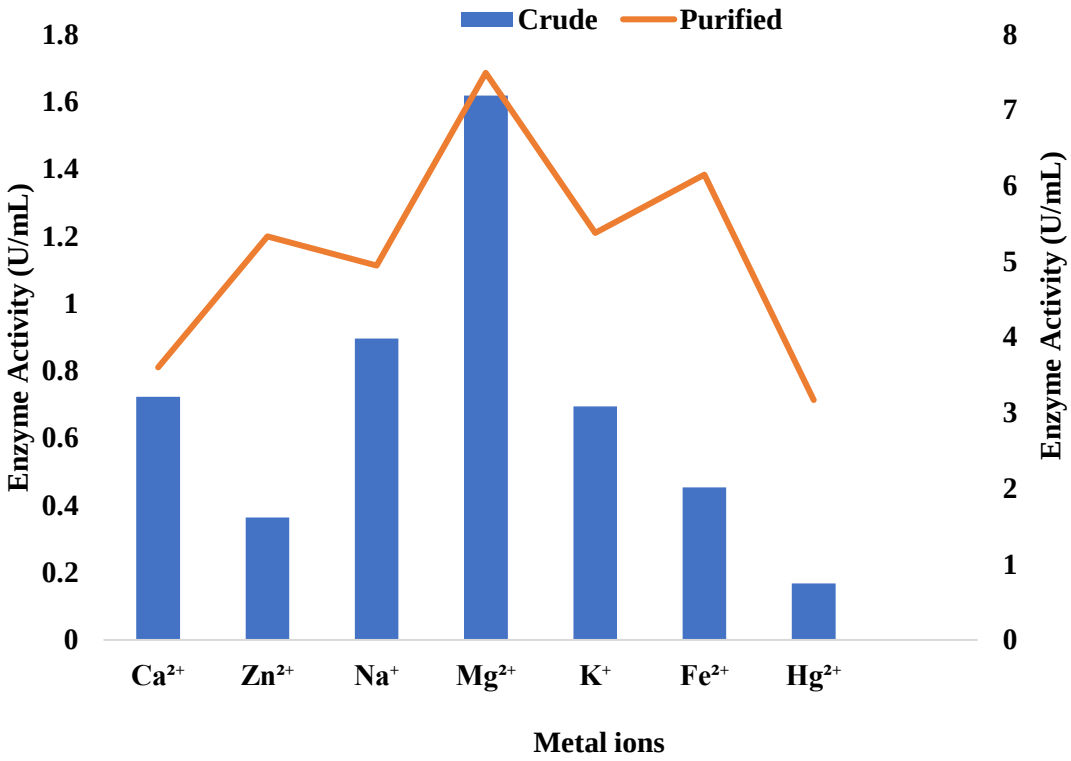


Figure 4.36: Effect of metal ions on activity of crude and purified L–asparaginase produced from Sp*hingobium yanoikuyae*

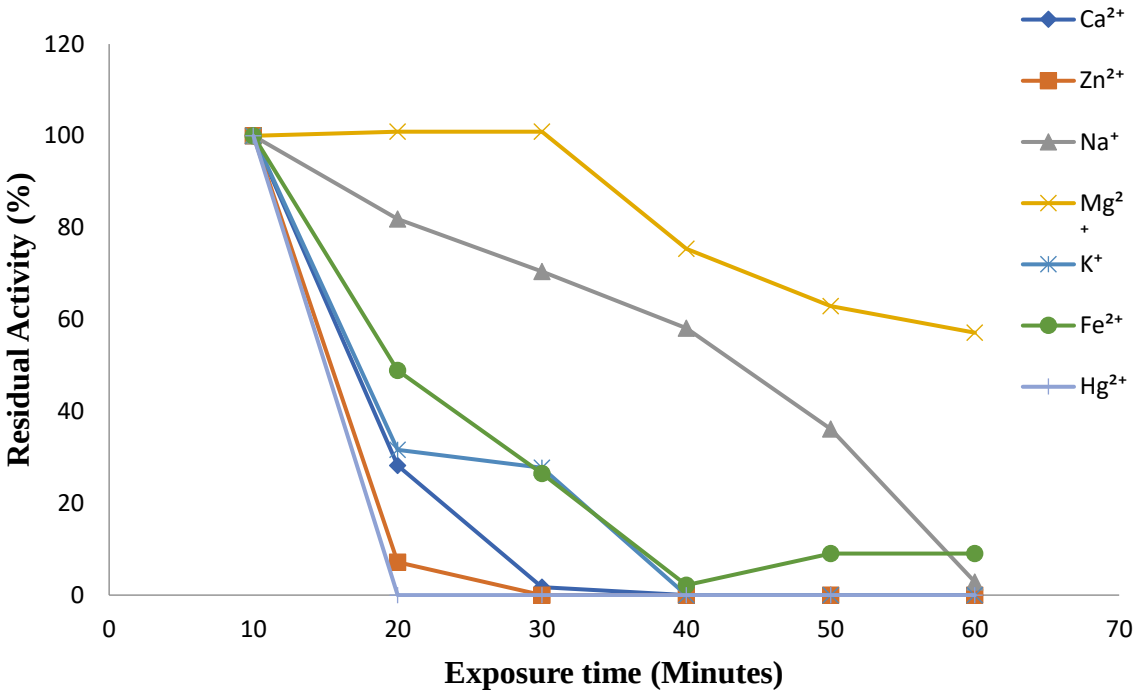


Figure 4.37: Effect of metal ions on stability of crude L–asparaginase from *Amycolatopsis japonica*

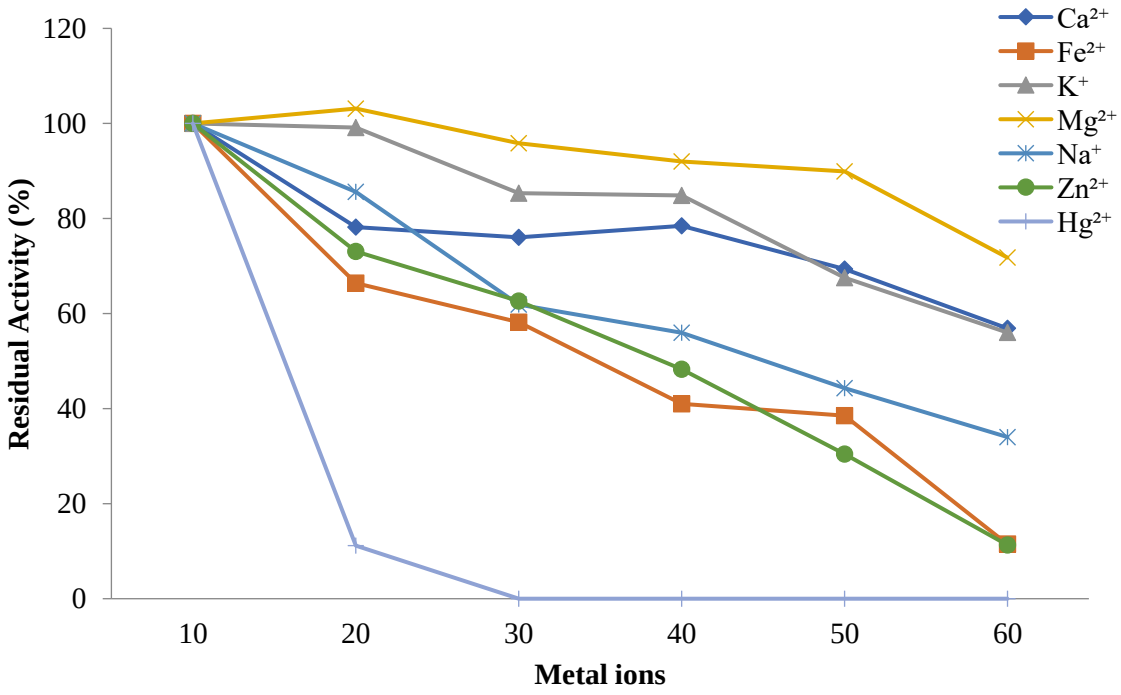


Figure 4.38: Effect of metal ions on stability of purified L–asparaginase from *Amycolatopsis japonica*

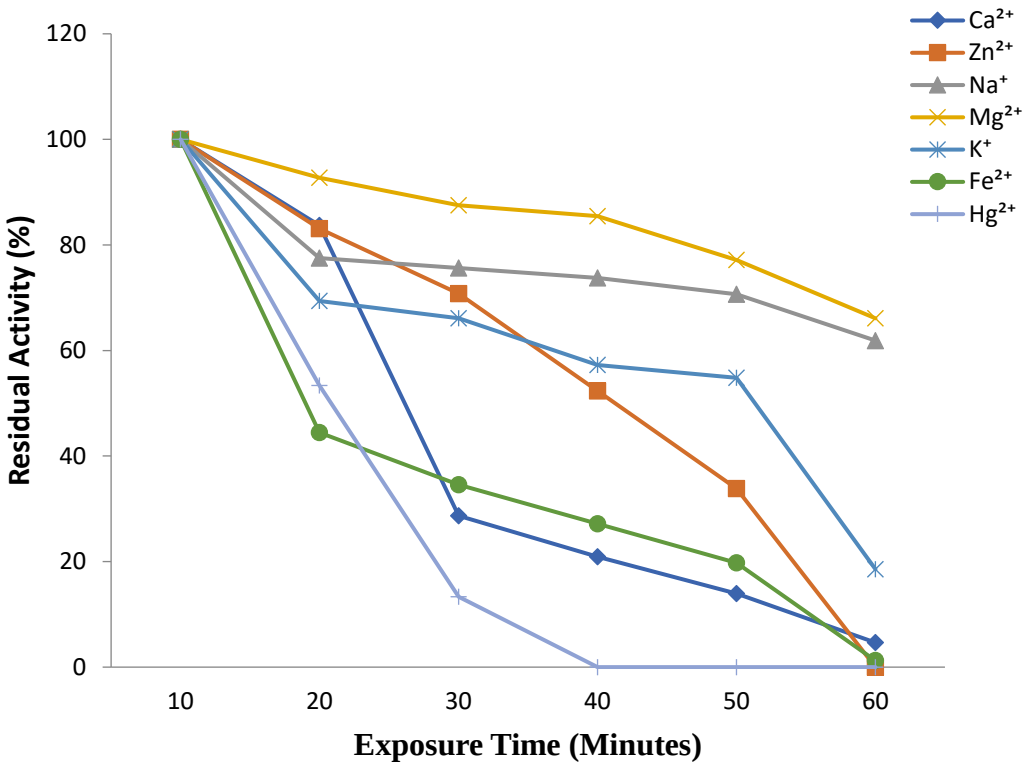


Figure 4.39: Effect of metal ions on stability of crude L–asparaginase from *Sphingobium yanoikuyae*

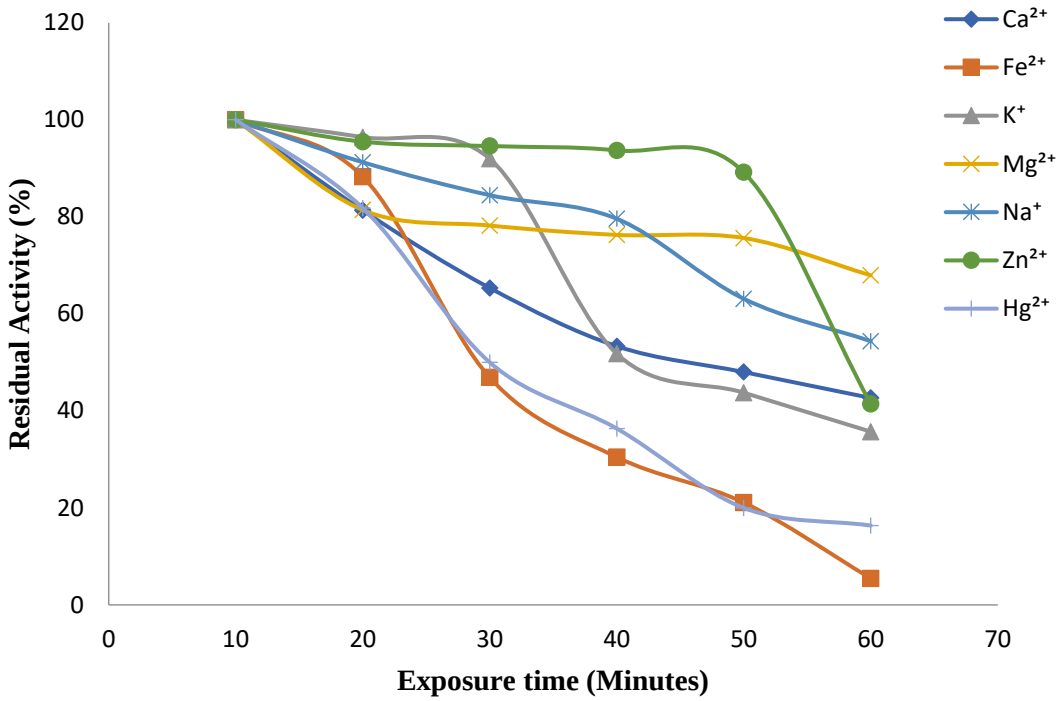


Figure 4.40: Effect of metal ions on stability of purified L–asparaginase from *Sphingobium yanoikuyae*

4.6.6. Effect of inducers and inhibitors on activity and stability of crude and purified L-asparaginase from *Amycolatopsis japonica and Sphingobium yanoikuyae*

The effect of *Amycolatopsis japonica and Sphingobium yanoikuyae* is shown in figures 4.41 to 4.45. Crude L-asparaginase from *Amycolatopsis japonica* showed the highest activity (6.46 U/mL) in the presence of EDTA and the minimum activity (0.56 U/mL) were recorded in the presence of SDS (Figure 4.41) with enzyme activity. EDTA acted as enhancer of crude L-asparaginase from *Amycolatopsis japonica* while others acted as inhibitors. Triton X-100, Tween 80 and EDTA acted as inducers for the purified L-asparaginase from *Amycolatopsis japonica.* The highest enzyme activity was in the presence of Triton-X-100, tween 80 and EDTA with the enzyme activity of 15.56 U/mL, 13.92 U/mL and 18.73 U/mL respectively. The maximum activity of the enzyme was found in the presence of EDTA with 18.73 U/mL and the lowest activity was found in the presence of ascorbic acid with enzyme activity of 4.89 U/mL.

The activity of crude L-asparaginase from *Sphingobium yanoikuyae* was enhanced by EDTA with the highest enzyme activity of 4.96 U/mL, the lowest activity was in the presence of sodium azide. SDS, Ascorbic acid and Urea acted as inhibitors of this enzyme (Figure 4.42). The purified enzyme from *Sphingobium yanoikuyae* was enhanced in the presence of Tween 80, Triton X 100 and EDTA. The maximum activity of the enzyme was found in the presence of Tween 80 with 16.66 U/mL and the lowest activity was found in the presence of Ascorbic acid with enzyme activity of 1.87 U/mL.

The crude enzyme from *Amycolatopsis japonica* showed no activity in the presence of ascorbic acid and almost 80% of it activity after 10 minutes of incubation in the presence of SDS and Urea. The enzyme was stable within 30minutes of incubation and retained 60% of its activity after which there was gradual reduction of activity up to 2% after 60 minutes of incubation in the presence of Triton X 100. Up to 92% stability of the enzyme was noticed after 40minutes of incubation in the presence of EDTA. A 50% reduction of the enzyme activity was noticed in the presence of sodum azide after 30 minutes of incubation (Figure 4.43). The purified L-asparaginase from *Amycolatopsis japonica* lost 60% of its activity in the presence

of urea after 20 minutes of incubation and by the end of 60 minutes the enzyme activity was reduced to 20%. There was total loss of activity in the presence of ascorbic acid after 20 minutes of incubation at 37^{o}C. In the presence of Triton X-100, the residual activity of the enzyme was 65% within 30 minutes of incubation. The enzyme showed good stability within 60 minutes of incubation and retained upto 60% of its activity in the presence of EDTA. Good stability was also observed in the presence of SDS after 50 minutes of incubation with minimum residual activity of 54% after 50 minutes of incubation (Figure 4.44).

The crude enzyme from *Sphingobium yanoikuyae* showed no stability in the presence of ascorbic acid, urea and SDS within 10minutes of incubation. The enzyme was stable within 20minutes in the presence of Triton X- 100 after which there was total loss of activity after 20 minutes of incubation. The enzyme lost 60% of its activity in the presence of tween80 after 20 minutes of incubation, after which there was total loss of its activity. In the presence of EDTA, 64% of the enzyzme activity was retained within 20 minutes of incubation Reduction in the enzyme stability was observed as the incubation progressed. A 60% of the enzyme stability was noticed in the presence of sodium azide after 40 minutes of incubation (Figure 4.45). In the presence of urea, gradual reduction in the activity of the purified L-asparaginase from *Sphingobium yanoikuyae* was noticed after 10 minutes of incubation and 77% lost of activity was recorded after 60 minutes of incubation.. There was 90% loss of enzyme activity after 20 minutes of incubation in the presence of ascorbic acid. In the presence of TritonX-100, Tween 80 and EDTA, the enzyme activity was enhanced with 14%, 18% and 3% respectively after 20 minutes of incubation, decrease in the enzyme stability with prolonged incubation time was observed. 60% of the enzyme activity was retained after 30 minutes of incubation in the presence of SDS (Figure 4.46).

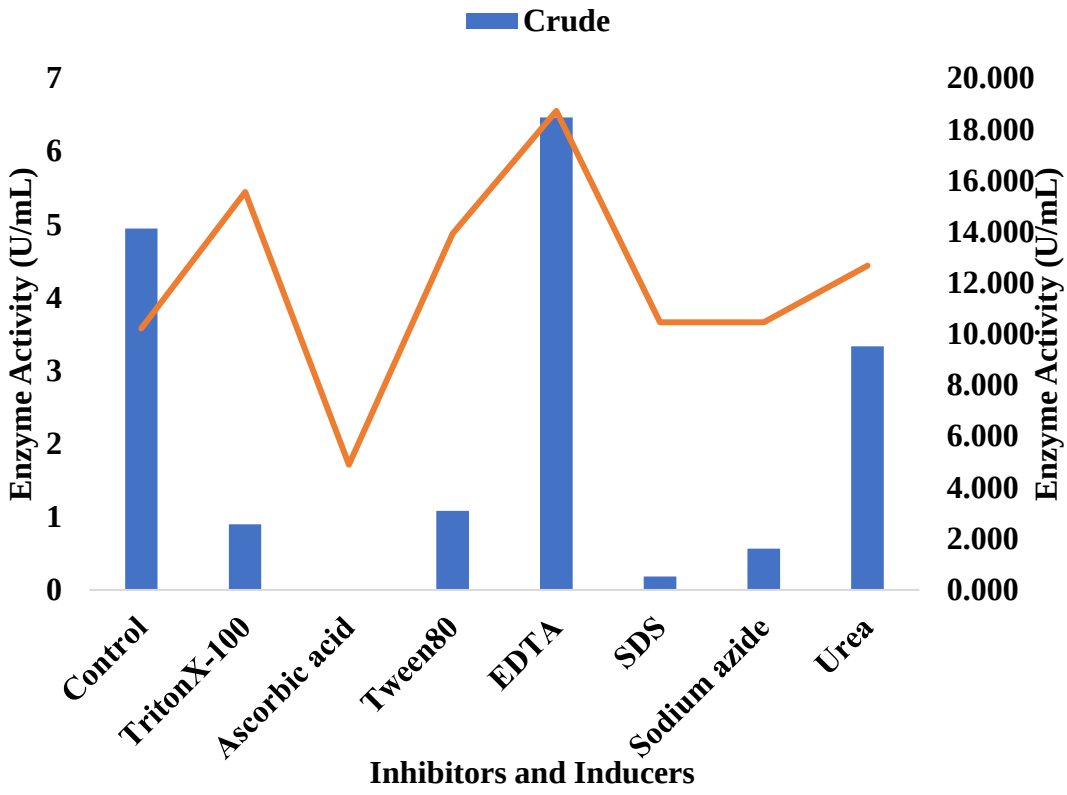


Figure 4.41: Effect of inhibitors and inducers on activity of crude and purified L–asparaginase produced from *Amycolatopsis japonica*

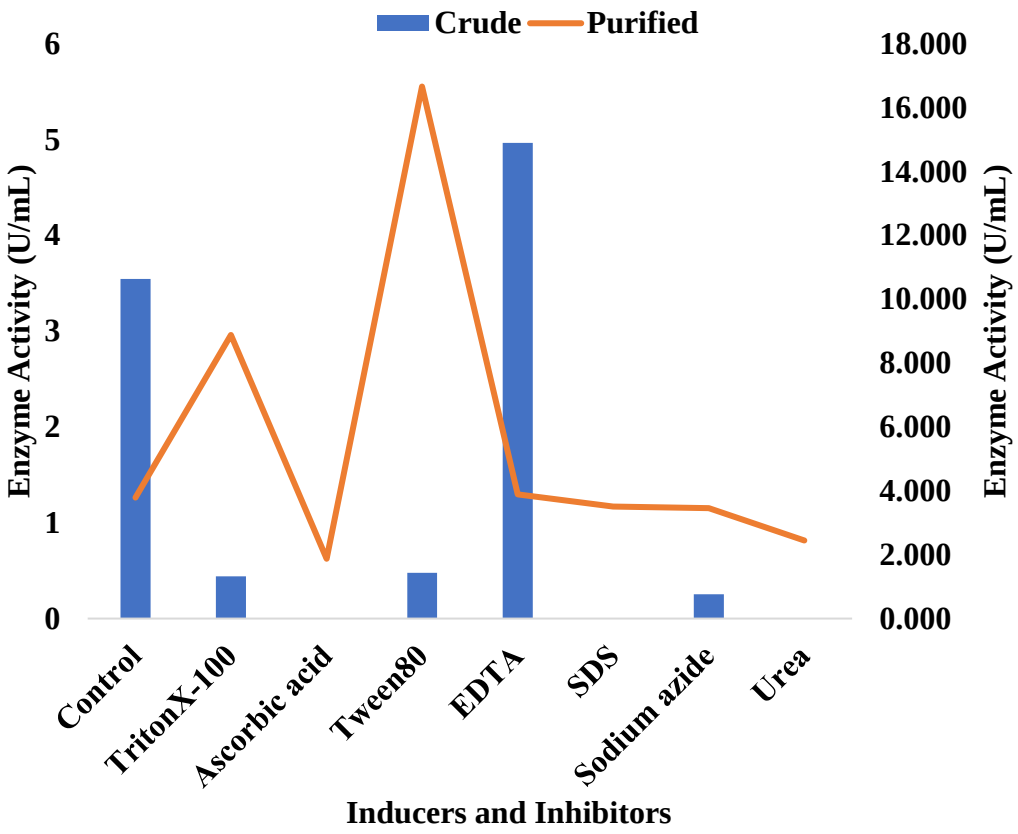


Figure 4.42: Effect of inhibitors and inducers on activity of crude and purified L–asparaginase produced from Sp*hingobium yanoikuyae.*

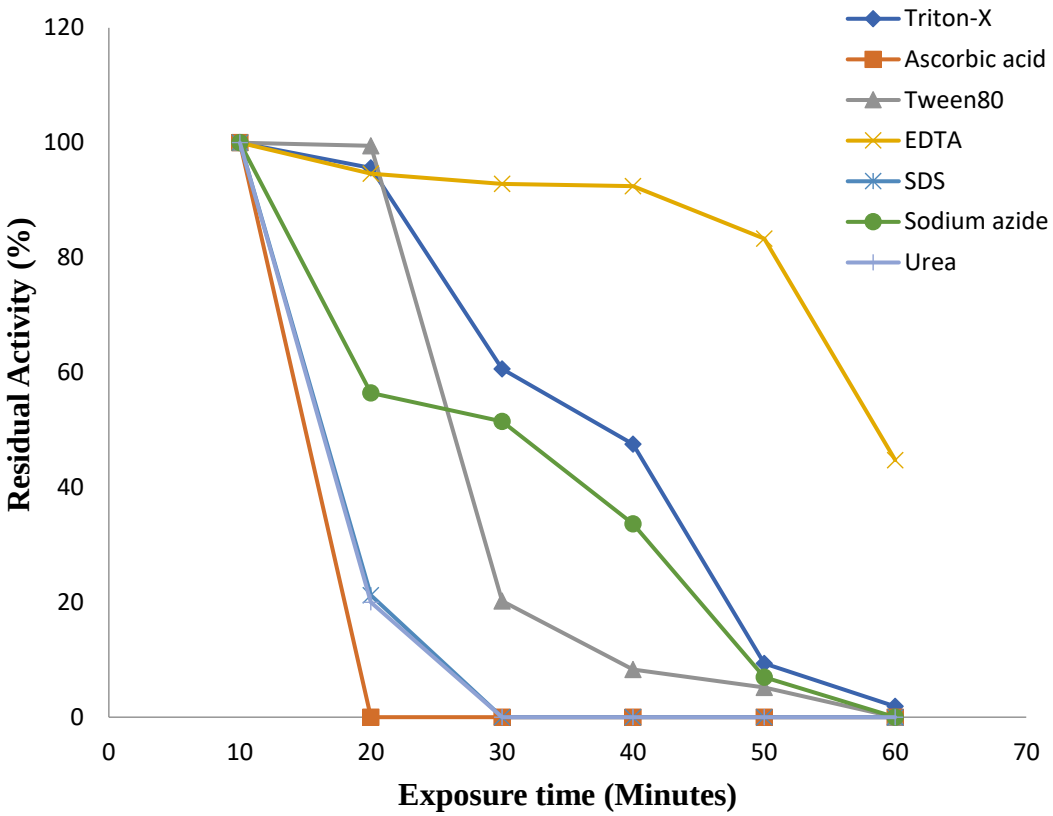


Figure 4.43: Effect of inhibitors and inducers on stability of crude L–asparaginase from *Amycolatopsis japonica*

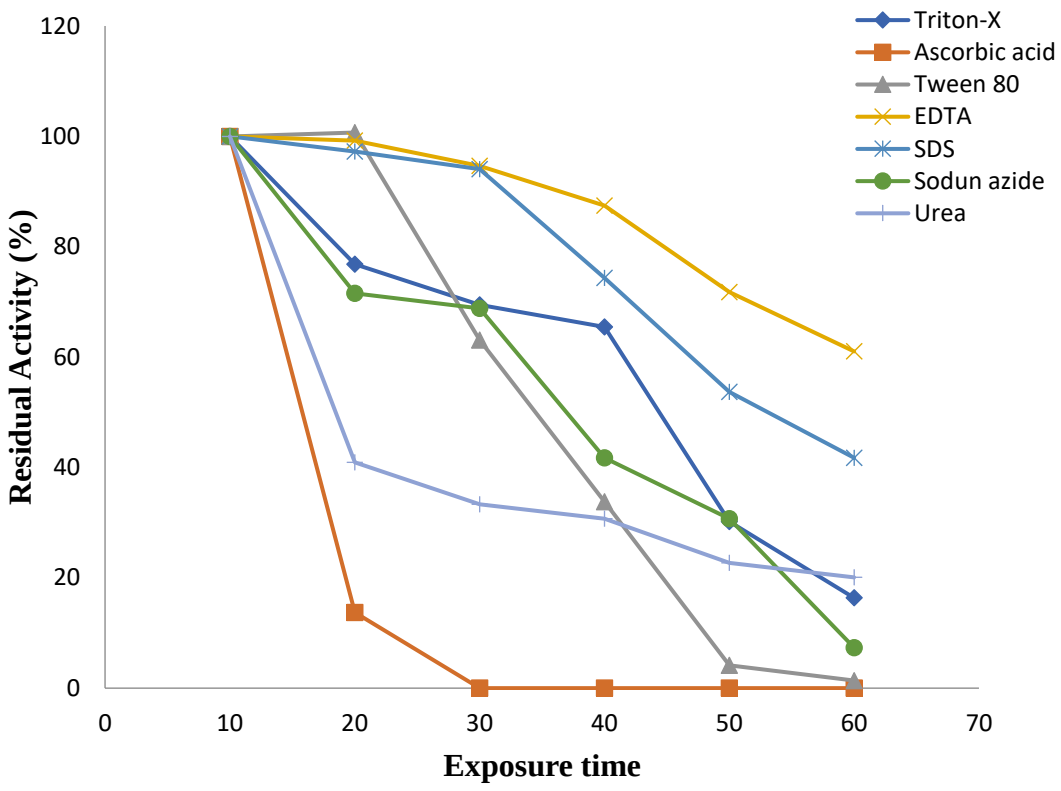


Figure 4.44: Effect of inhibitors and inducers on stability of purified L–asparaginase from *Amycolatopsis japonica*

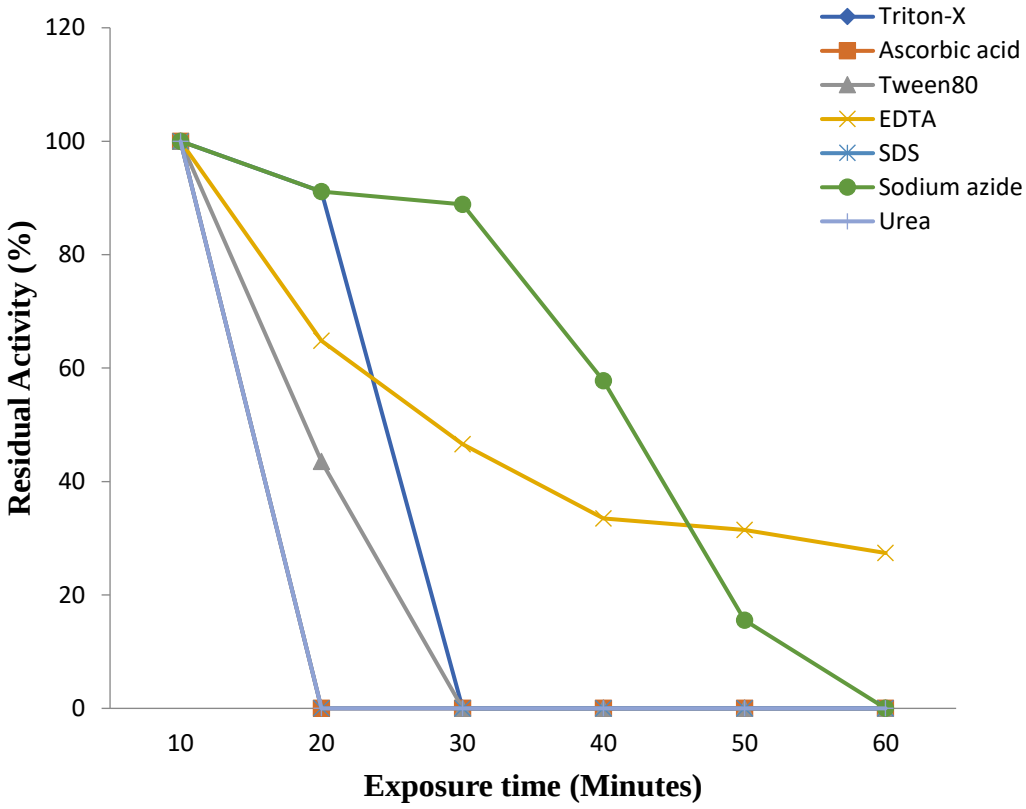


Figure 4.45: Effect of inhibitors and inducers on stability of crude L–asparaginase from *Sphingobium yanoikuyae*

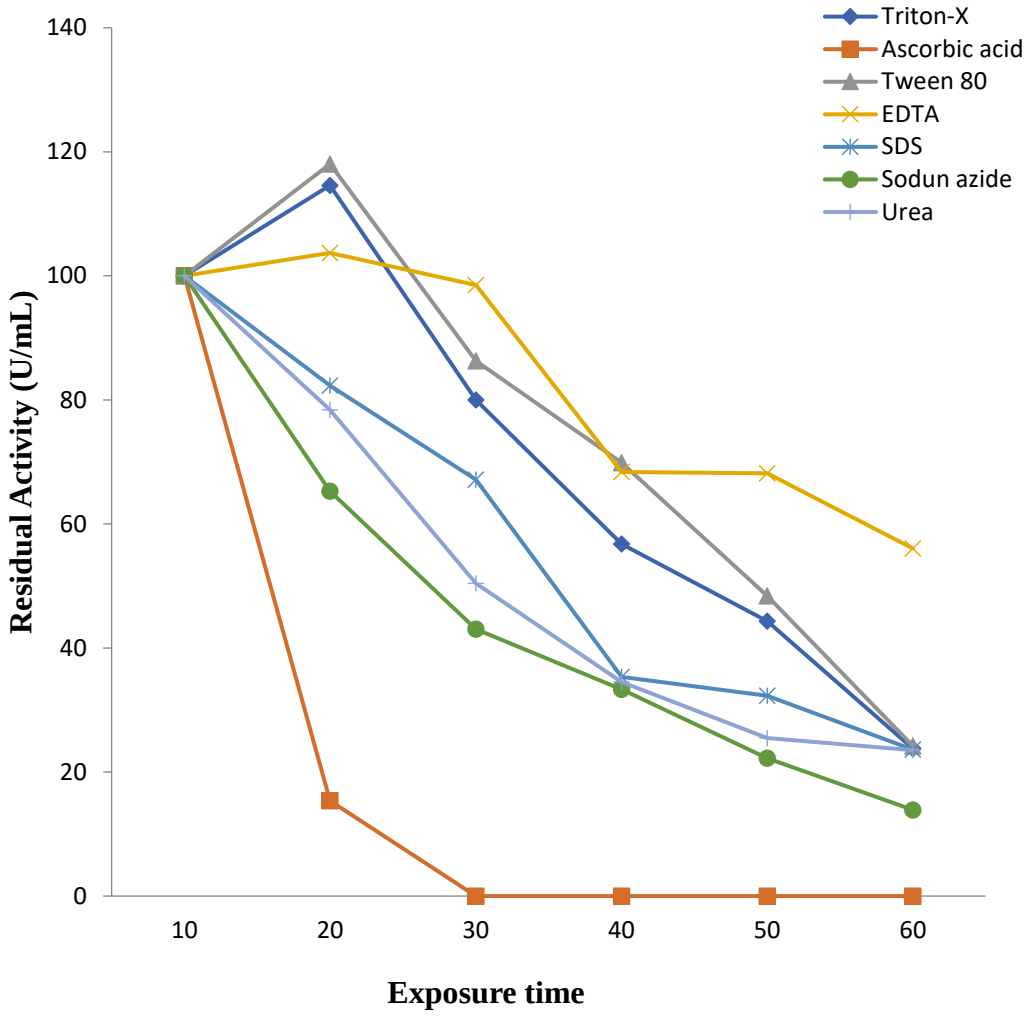


Figure 4.46: Effect of inhibitors and inducers on stability of purified L–asparaginase from *Sphingobium yanoikuyae*

4.6.7. Effect of amino acid on activity and stability of crude and purified L-asparaginase from *Amycolatopsis japonica and Sphingobium yanoikuyae.*

The effect of different amino acid on the activity of crude and purified L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* is as recorded in Figure 4.47 to 4.52. The crude L-asparaginase from *Amycolatopsis japonica* showed higher activity in the presence of L-phenylalanine, L-asparagine and L-aspartic acid. The highest activity was noticed in the presence of L-asparagine with 4.8 U/mL, while lowest enzyme activity was noticed in the presence of L-glutamine with 0.059 U/mL. The purified L-asparaginase from *Amycolatopsis japonica* showed the lowest activity in the presence of L-glutamine compared to the control and other amino acids studied, while its showed higher activity in the presence of L–aspartic acid and L–phenylalanine. The highest enzyme activity was noticed in the presence L-asparagine with enzyme activity of 37.60 U/mL and the lowest activity of 4.08 U/mL in the presence of L-glutamine (Figure 4.47).

The crude L-asparaginase from *Sphingobium yanoikuyae* showed highest activity in the presence of L-asparagine with enzyzme activity of 5.33 U/mL, while lowest enzyme activity was recorded in the presence of L-glutamine with 0.56 U/mL. The purified L-asparaginase from *Sphingobium yanoikuyae* gave highest activity in the presence of L–phenylalanine with enzyme activity of 38.17 U/mL while the lowest activity was in the presence of L-arginine with enzyme activity of 4.56 U/mL (Figure 4.48).

The activity of the crude L-asparaginase from *Amycolatopsis japonica* was reduced with 56% after 60 minutes incubation in the presence of L–aspartic acid. Total loss of activity of the enzyme was observed after 20 minutes of incubation in the presence of L-glutamine. The enzyme retained 70% and 78% of its activity in the presence of L–phenylalanine and L-asparagine respectively throughout the incubation period of 60 minutes. There was total loss of activity in the presence of L-arginine after 20 minutes of incubation (Figure 4.49). The enzyme activity of purified L-asparaginase from *Amycolatopsis japonica* reduced drastically in the presence of L-arginine as the incubation progressed. The enzyme showed good stability in the presence of L-phenylalanine with residual activity of 81% after 60 minutes of incubation. There was reduction in the activity of the enzyme with

increase in time of incubation in the presence of L–asparagine. Also, the presence of L–aspartic acid decreases the activity of the enzyme up to 50% after incubation for 60 minutes. There was total loss of activity after 30 minutes in the presence of L glutamine (Figure 4.50).

The crude L-asparaginase from *Sphingobium yanoikuyae* retained 50% of its activity after incubation for 60 minutes of incubation in the presence of L–aspartic acid and L-asparagine. A 18% of the enzyme activity was enhanced after 20 minutes of incubation in the presence of L-phenyalanine, after which there was gradual reduction up to 20% activity after 60 minutes of incubation. Up to 60% loss of activity of the enzyme was observed after 30 minutes of incubation in the presence of L- glutamine and L-arginine (Figure 4.51).

The enzyme activity of purified L-asparaginase from *Sphingobium yanoikuyae* was reduced to 65% in the presence of L-aspartic acid after 30 minutes of incubation. The enzyme showed good stability of in the presence of L-phenylalanine and L-arginine with residual activity of 70% after 50 minutes of incubation. The enzyme retained 68% of its activity in the presence of L-asparagine after 60 minutes of incubation. The enzyme retained 50% of it activity in the presence of L-glutamine after 60 minutes of incubation (Figure 4.52).

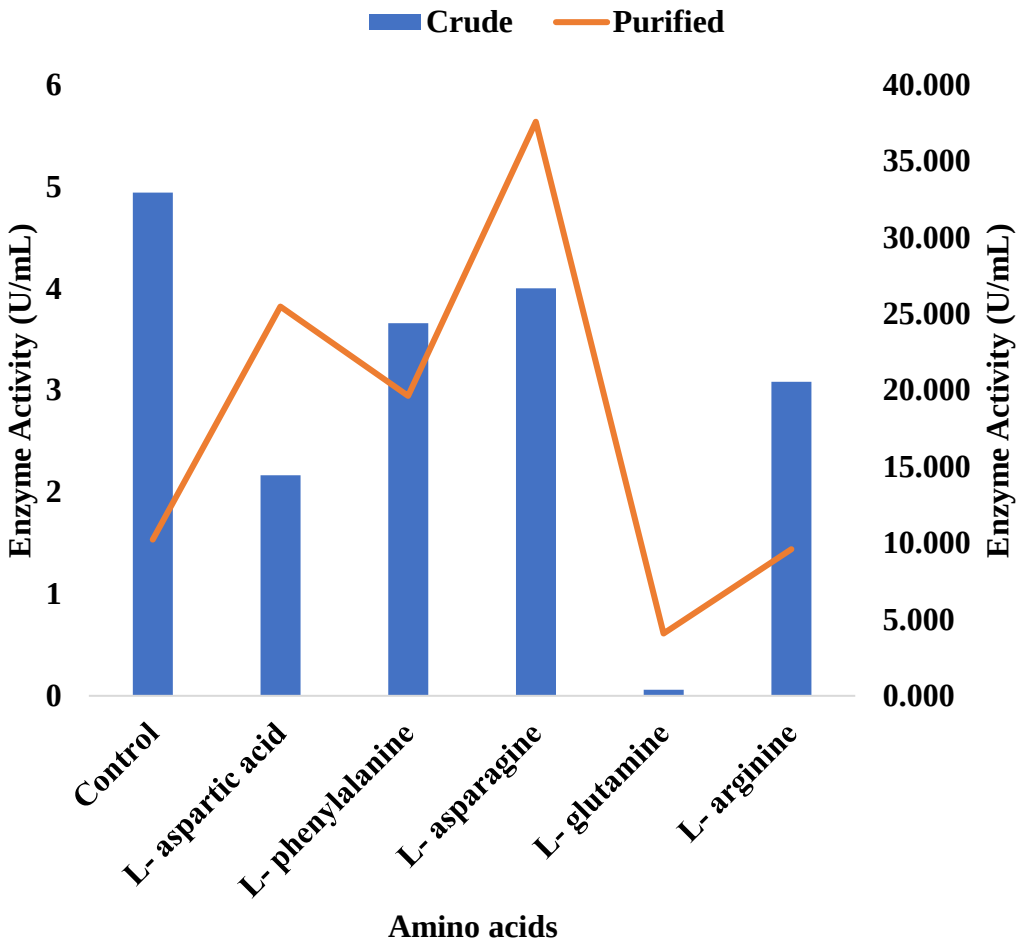


Figure 4.47: Effect of amino acids on activity of crude and purified L–asparaginase produced from *Amycolatopsis japonica.*

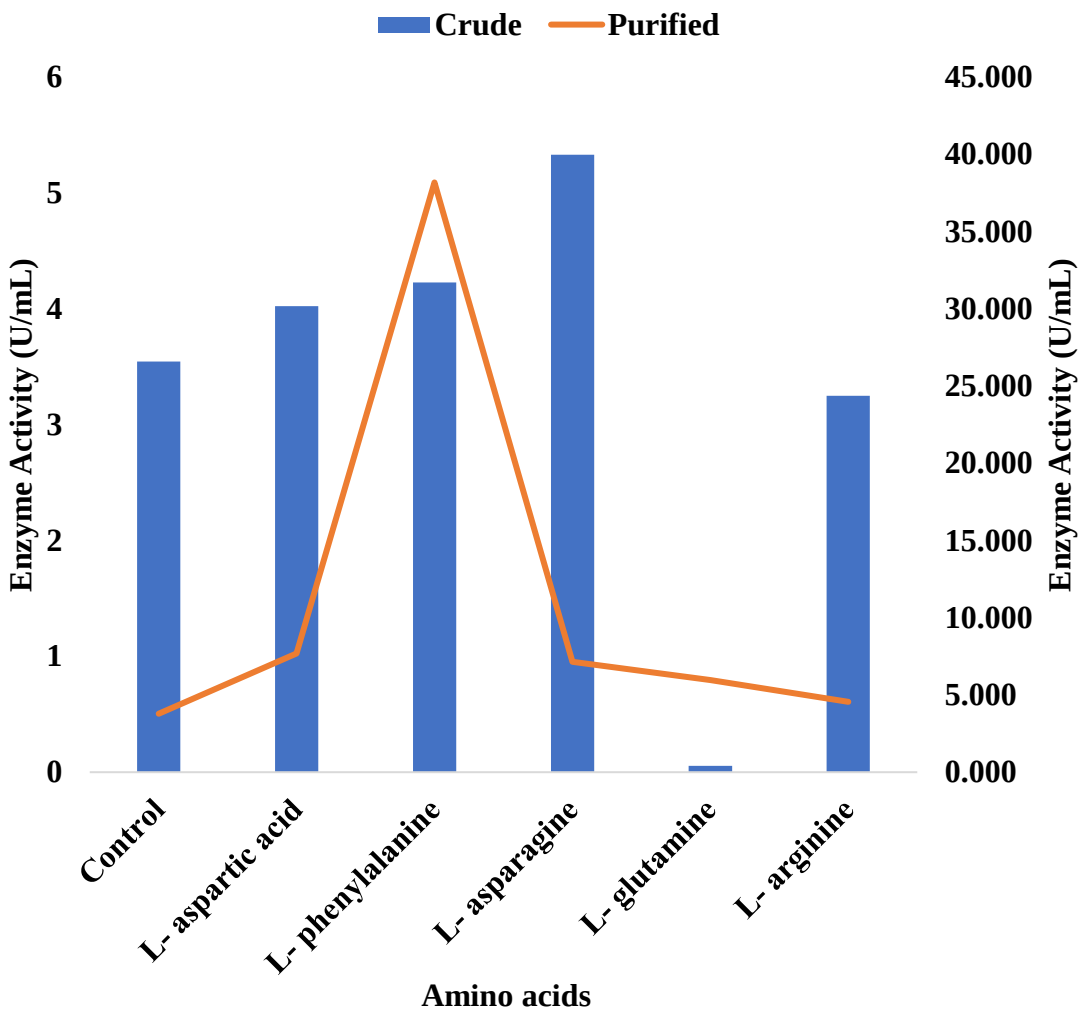


Figure 4.48: Effect of amino acids on activity of crude and purified L–asparaginase produced from Sp*hingobium yanoikuyae.*

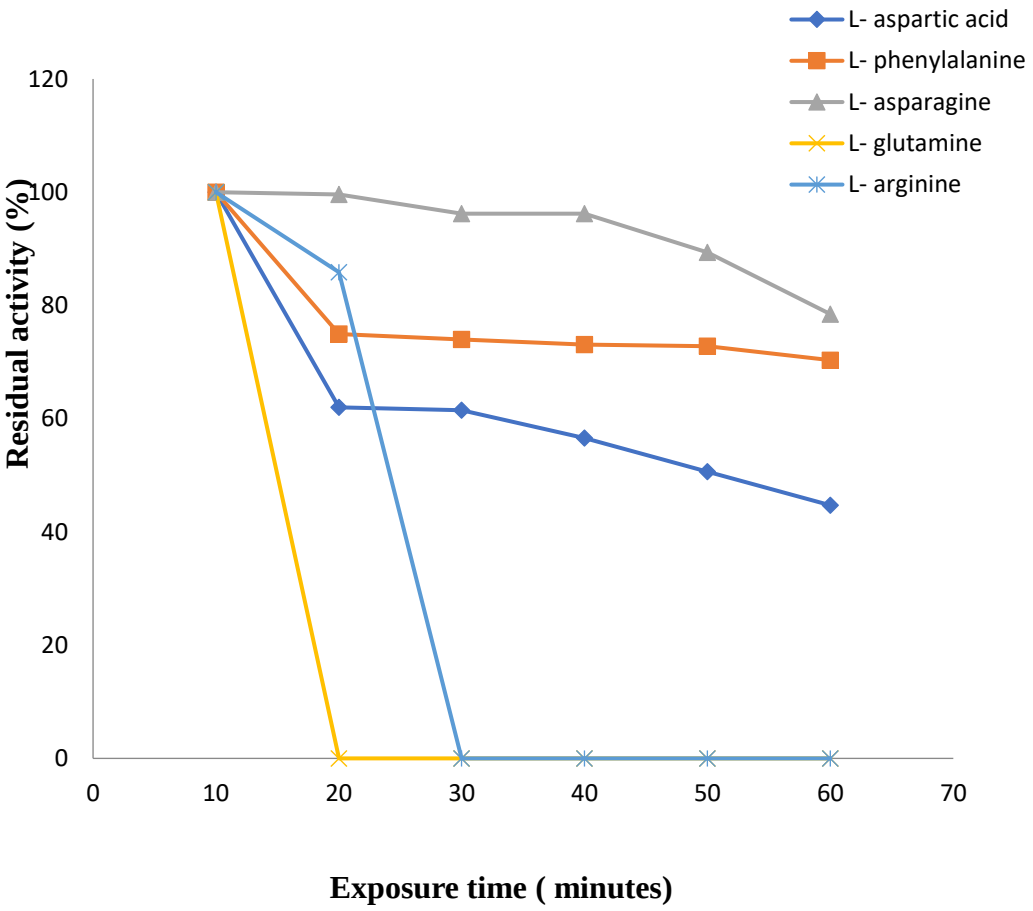


Figure 4.49: Effect amino acids on stability of crude L–asparaginase from *Amycolatopsis japonica.*

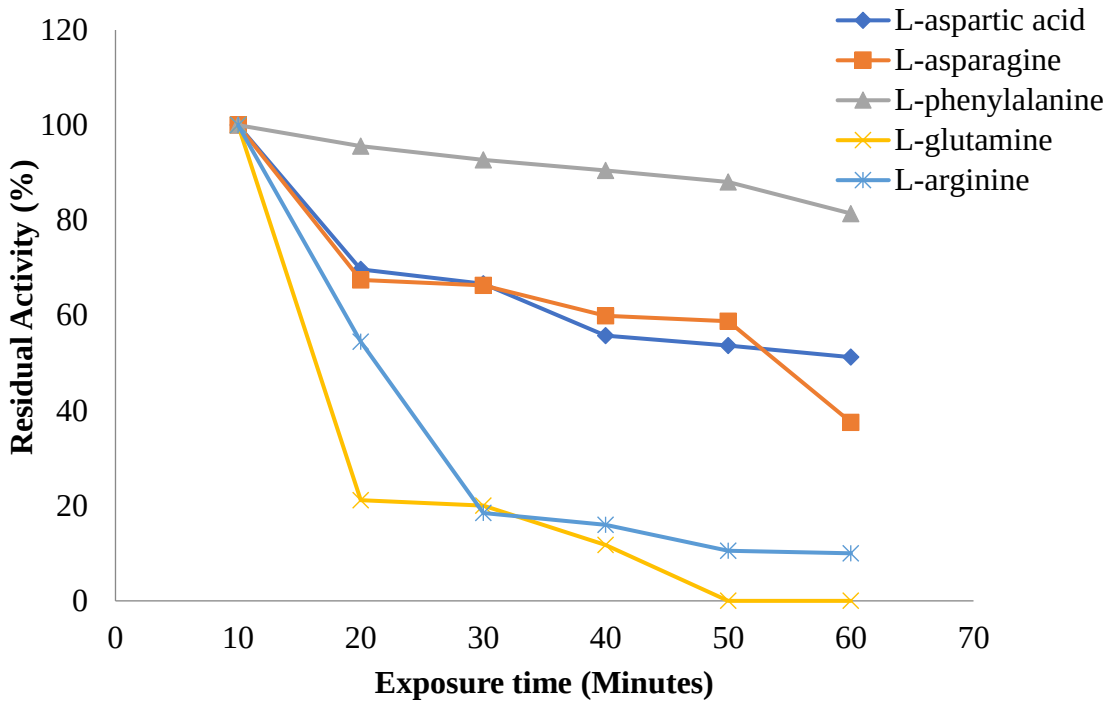


Figure 4.50: Effect of amino acids on stability of purified L–asparaginase from *Amycolatopsis japonica*

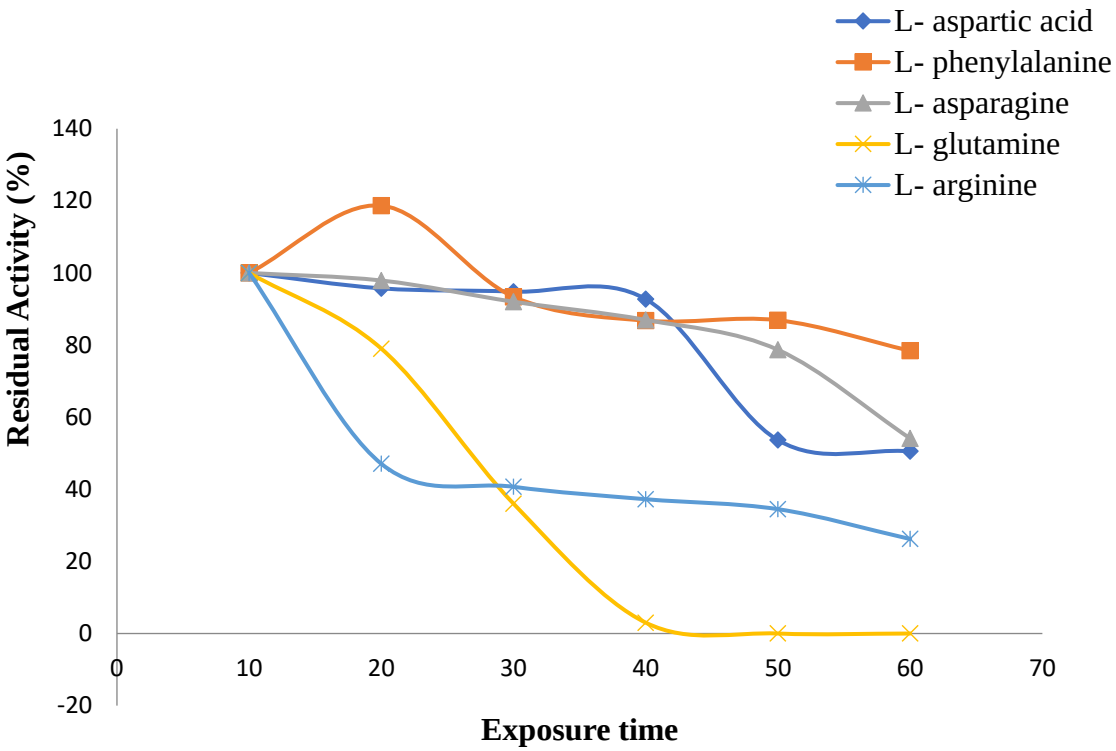


Figure 4.51: Effect of amino acids on stability of crude L–asparaginase from *Sphingobium yanoikuyae*

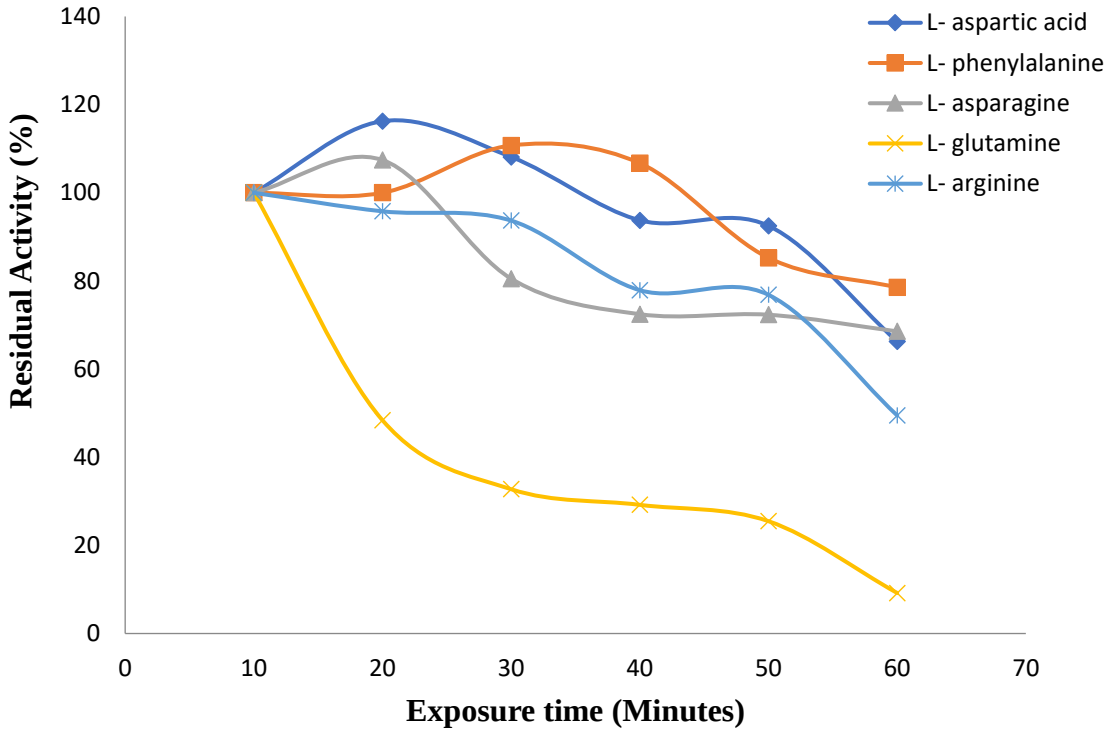


Figure 4.52: Effect of amino acids on stability of purified L–asparaginase from *Sphingobium yanoikuyae*

4.6.8. Effect of different enzyme concentration on activity and stability of crude and purified L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae.*

Effect of different enzyme concentrations on activity and stability of crude and purified L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* are as shown in Figure 4.53- 4.58. For the crude L-asparaginase from *Amycolatopsis japonica,* increase in the concentration of the enzyme led to an increase in the activity of the enzyme. The highest enzyme activity was recorded when enzyme assay was done with 0.4 mL enzyme with enzyme activity of 11.97 U/mL. There was a slight reduction of about 0.5 U/mL in the enzyme activity when 0.5 mL was used. The lowest enzyme activity was recorded when 0.1 mL was used in the assay mixture. The highest enzyme activity (17.24 U/mL) of the purified L-asparaginase from *Amycolatopsis japonica* was recorded in the assay mixture containing 0.5mL enzyme concentration, while the lowest activity (4.65 U/mL) was recorded with assay mixture with 0.1 mL enzyme concentration (Fig 4.53).

The crude L-asparaginase from *Sphingobium yanoikuyae* gave the highest enzyme activity (4.66 U/mL) when enzyme assay was done with 0.5 mL. The lowest activity was recorded when 0.1 mL was used in the assay mixture with enzyme activity of 0.22 U/mL (Figure 4.63). The purified enzyme from *Sphingobium yanoikuyae* gave the highest enzyme activity in the assay mixture with 0.4 mL enzyme concentration with enzyme activity of 11.23 U/mL, while the lowest enzyme activity was recorded with assay mixture with 0.3 mL enzyme with enzyme activity of 4.85 U/mL (Figure 4.54).

An 86% residual activity of crude L-asparaginase from *Amycolatopsis japonica* was recorded after 60 minutes of incubation at 37^{o}C when 0.5 mL enzyme concentration was used for the assay. The enzyme lost up to 90% of its activity after 60 minutes of incubation with assay mixture with 0.1 mL and 0.2 mL enzyme (Figure 4.55). Decrease in the enzyme activity of purified L-asparaginase from *Amycolatopsis japonica* was recorded with increase in incubation period in assay mixture with 0.1 mL enzyme as 69% of the enzyme activity was retained after 60 minutes of incubation. The enzyme lost up to 60% of its activity after 40 minutes of incubation in an assay mixture with 0.2 mL and 0.3 mL and showed higher

stability in the assay mixture with 0.4 and 0.5 mL enzyme as its retained 60% of its activity after 50 minutes of incubation (Fig 4.56).

The crude L-asparaginase from *Sphingobium yanoikuyae* retained up to 75%, 78% and 86% of its activity in the assay mixture with 0.3 mL, 0.4 mL and 0.5 mL respectively after 60 minutes of incubation. The enzyme lost 90% of it activity in the assay mixture with 0.1 mL enzyme concentration after 50 minutes of incubation and total lost of the activity was observed after 60 minutes (Figure 4.57). There was reduction in the activity of the purified L-asparaginase from *Sphingobium yanoikuyae* with up to 45% reduction of activity after 30 minutes of incubation and by the end of 60 minutes incubation, the activity has reduced to 73% in assay mixture with 0.1 mL of enzyme. The activity of the enzyme was enhanced with 10% in an assay mixture with 0.5 mL after 20 minutes of incubation and 77% of its activity was retained after 60 minutes of incubation in an assay mixture with 0.5 mL enzyme (Fig 4.58).

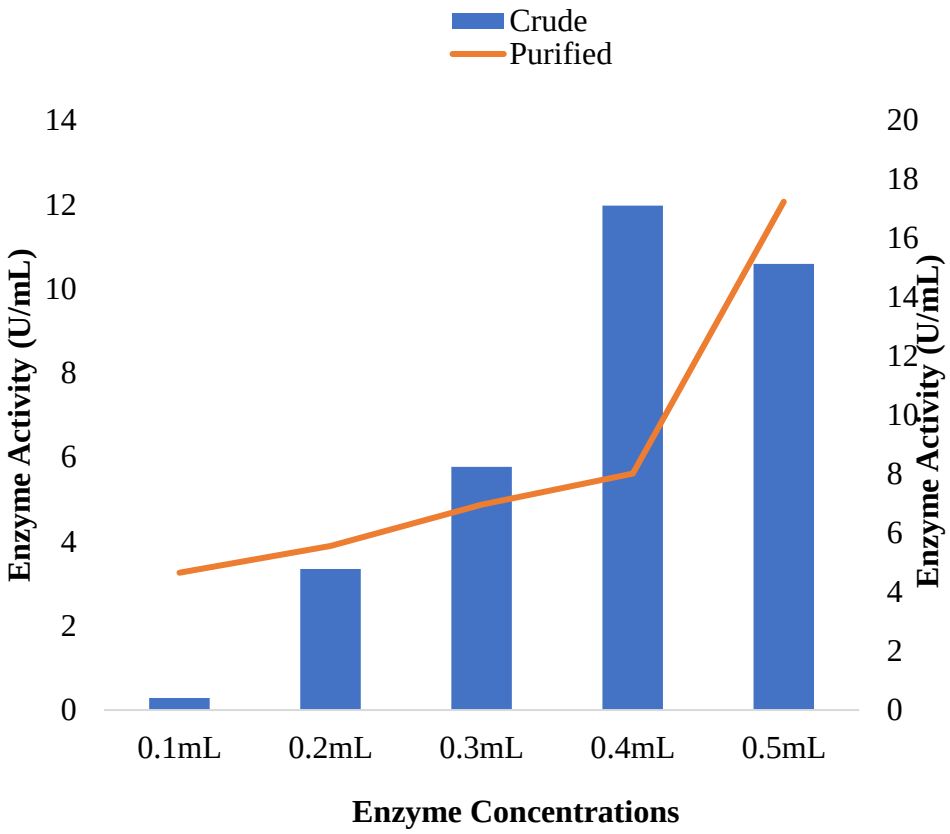
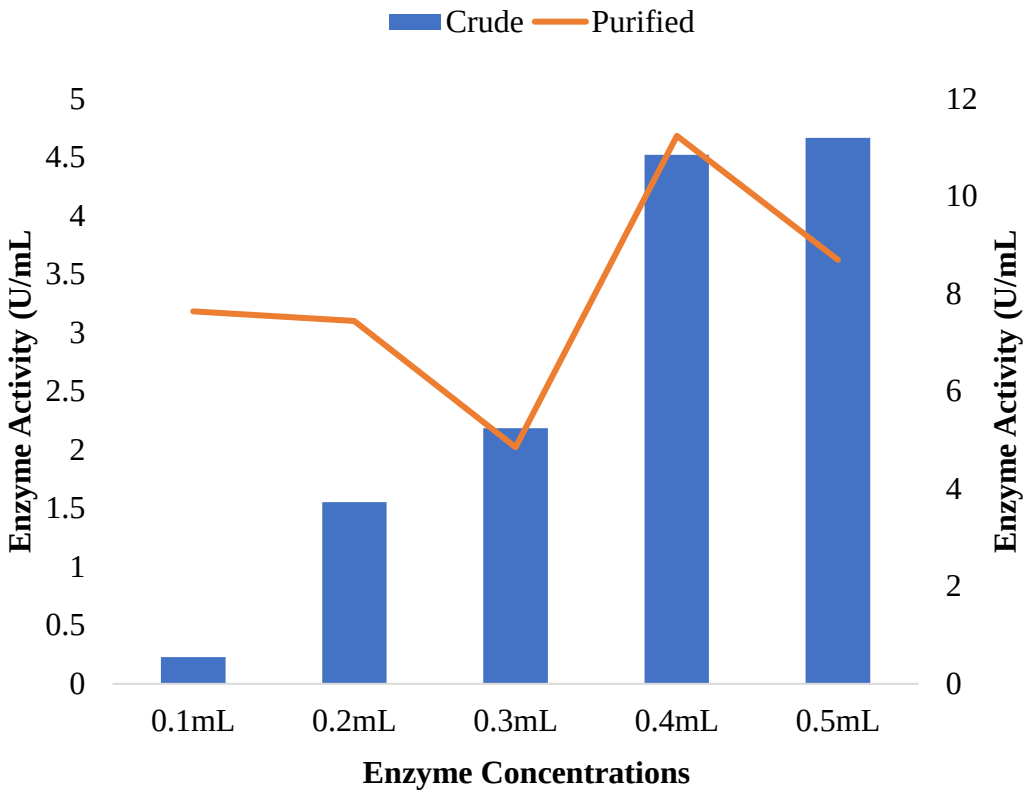


Figure 4.53: Effect of enzyme concentration on activity of crude and purified L–asparaginase produced from *Amycolatopsis japonica.*



4.54: Effect enzyme on activity of purified L–asparaginase produced from *Amycolatopsis japonica and* Shingobium *yanoikuyae*

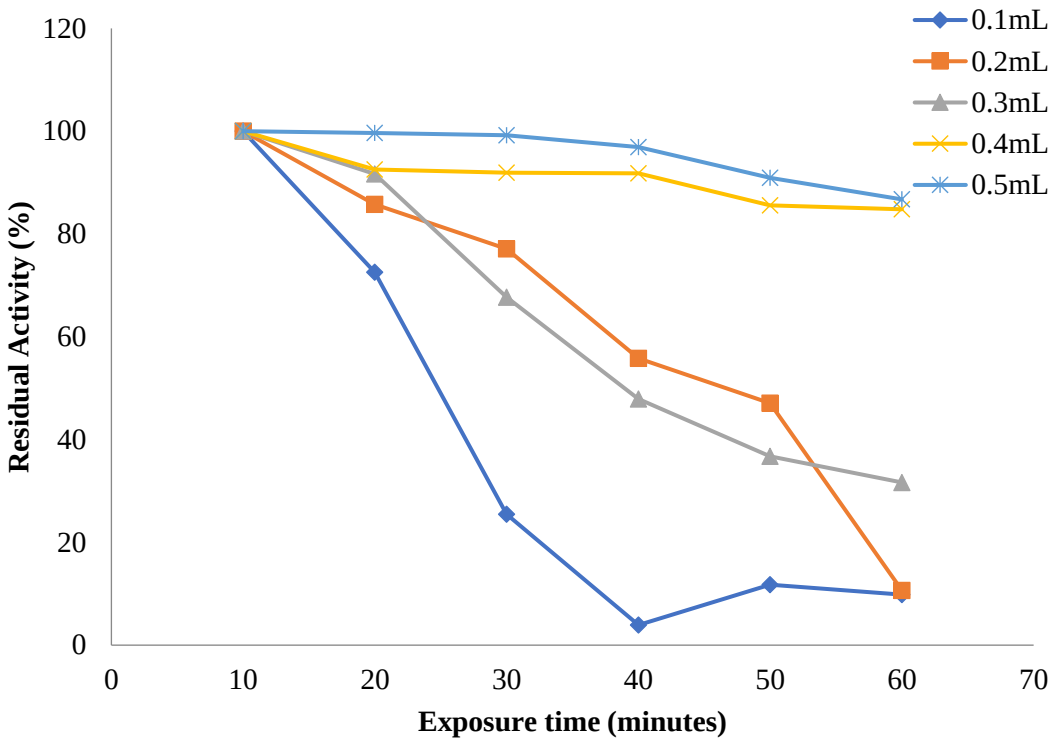


Figure 4.55: Effect of enzyme concentrations on stability of crude L–asparaginase from *Amycolatopsis japonica.*

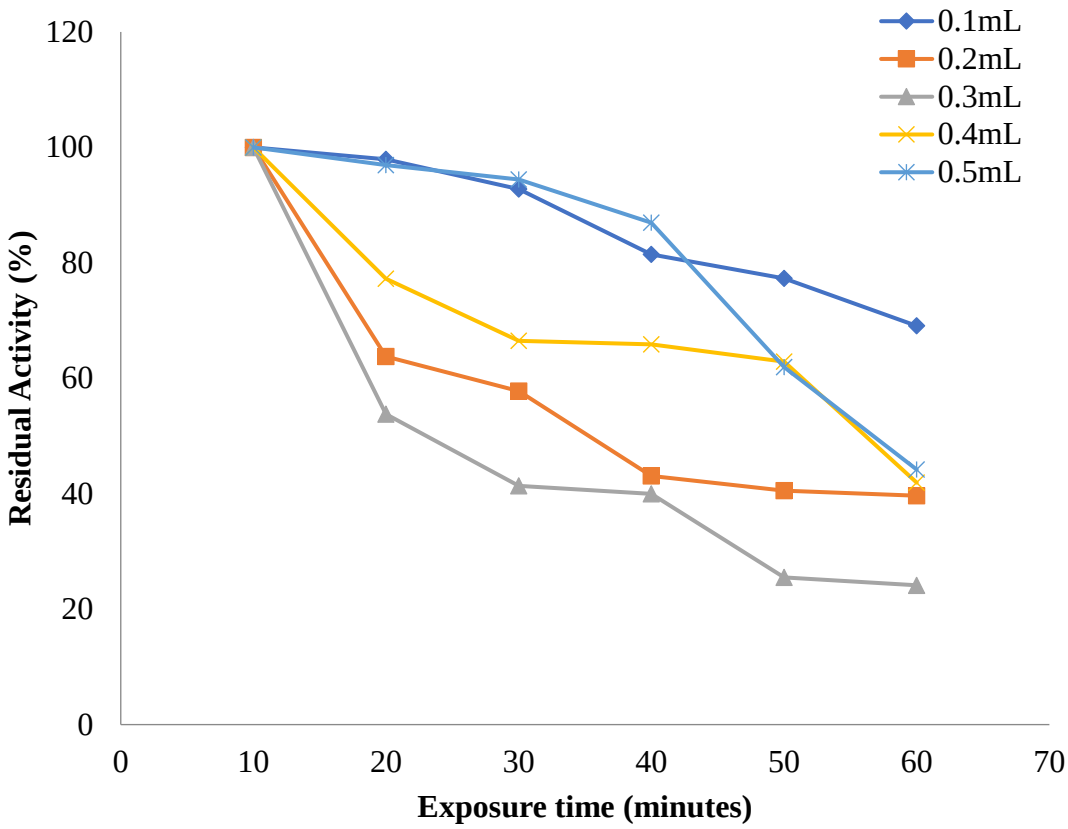


Figure 4.56: Effect of enzyme concentrations on stability of purified L–asparaginase from *Amycolatopsis japonica*

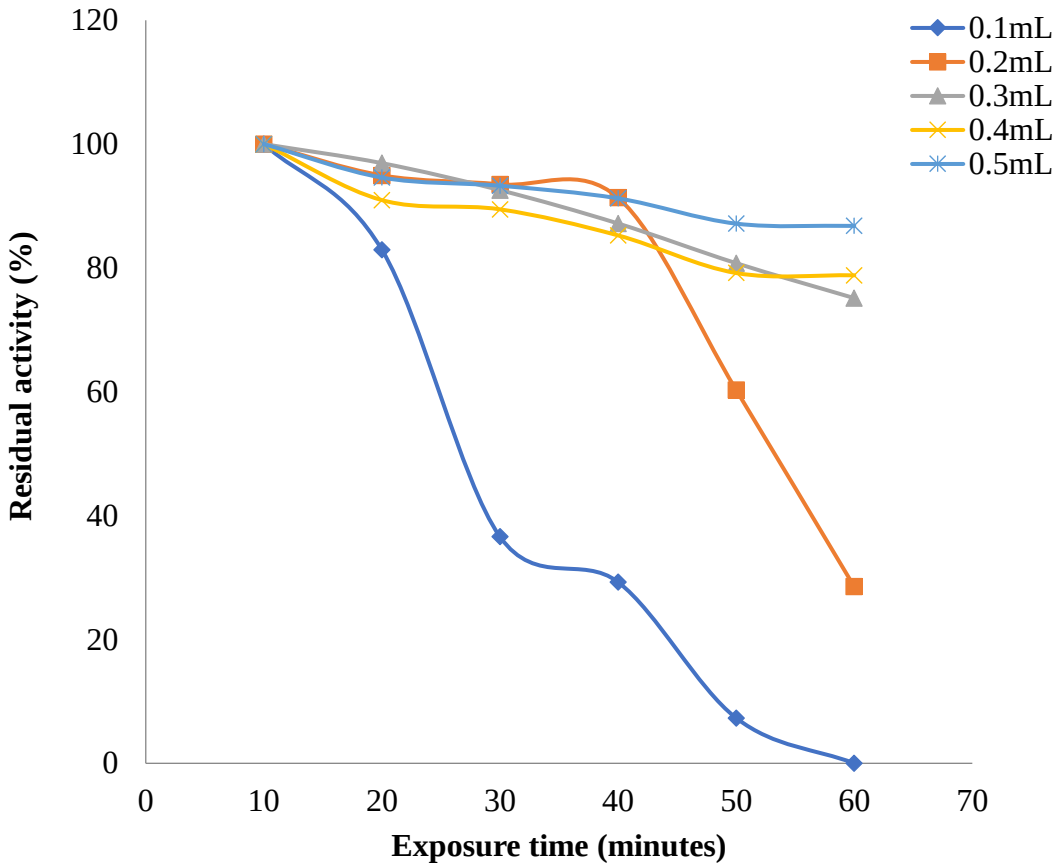


Figure 4.57: Effect of enzyme concentrations on stability of crude L–asparaginase from *Sphingobium yanoikuyae*

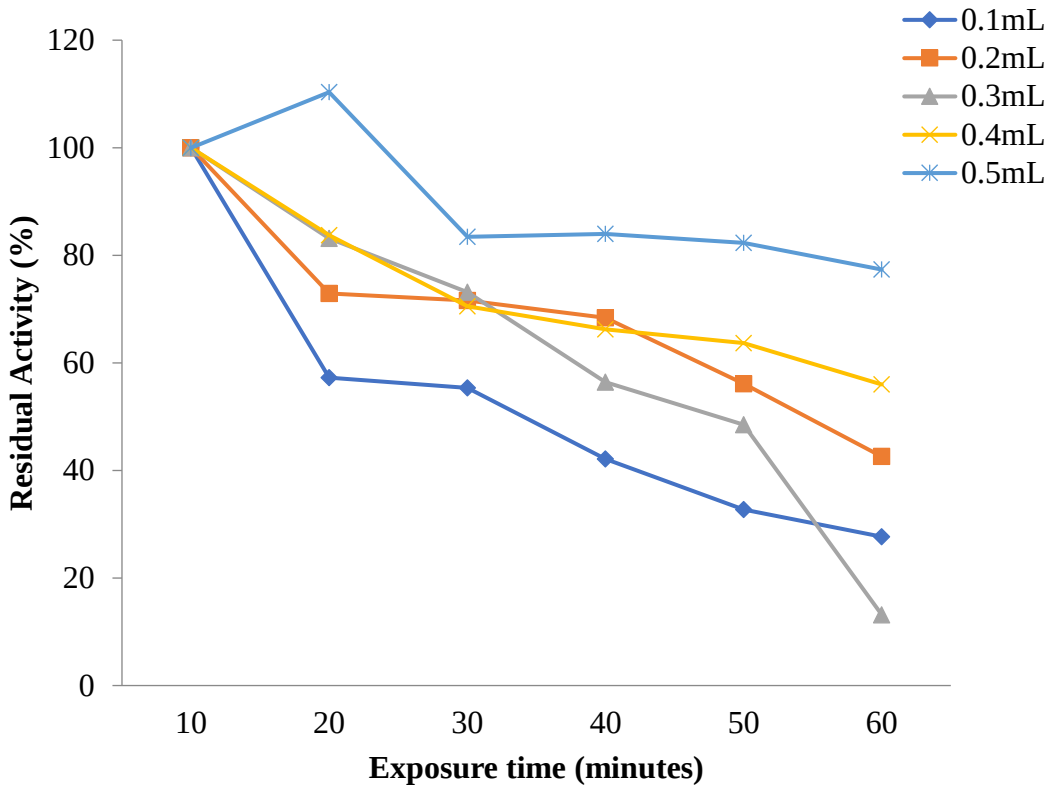


Figure 4.58: Effect of enzyme concentration on stability of purified L–asparaginase from *Sphingobium yanoikuyae*

4.6.9. Effect of substrate concentration on activity of crude and purified L-asparaginase from *Amycolatopsis japonica and Sphingobium yanoikuyae*

Use of different concentrations of the L–asparagine (0.4 to 2.0 mM) showed that the maximum substrate concentration that gave the highest enzyme activity for the crude L-asparaginase from *Amycolatopsis japonica* was 1.6 mM with the value of 3.04 U/mL (Figure 4.59). There was a gradual increase in L–asparginase activity from 0.4 mM to 1.6 mM after which there was a gradual decrease in the activity. The maximum enzyme activity (17.76 U/mL) of purified L-asparaginase from *Amycolatopsis japonica* was recorded when 0.8mM substrate concentration was used and the lowest activity (13.78 U/mL) was when 2.0mM was used.

The maximum substrate concentration that gave the highest enzyme activity activity for the crude L– asparaginase from *Sphingobium yanoikuyae* was 1.6 mM with the activity of 1.95 U/mL and the lowest activity (0.52 U/mL) was when 0.4 mM was used. Purified L-asparaginase from *Sphingobium yanoikuyae* showed maximum activity (17.76 U/mL) when 1.2 mM substrate was used and the lowest activity (7.63 U/mL) was 0.4 mM substrate was used (Figure 4.60)

The Michaelis constant (k_m) value and Maximum velocity (V_{max}) value of the crude and purified L-asparaginase from *Amycolatopsis japonica* as given in figure (4.61 and 4.63) were 7.87 mM and 2.57 U/mL, and 0.13 mM and 0.43 U/mL respectively. The km value and Vmax value of the crude and purified L-asparaginase from *Sphingobium yanoikuyae* were 4.45 mM and 6.34 U/mL and 0.37 mM and 0.32 U/mL respectively.

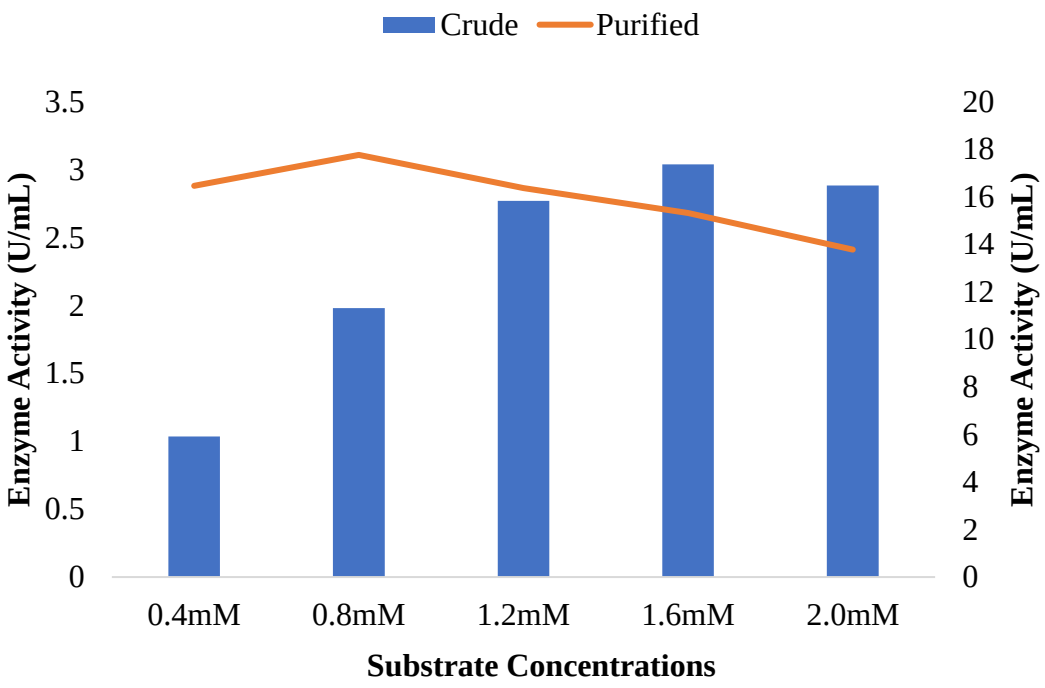
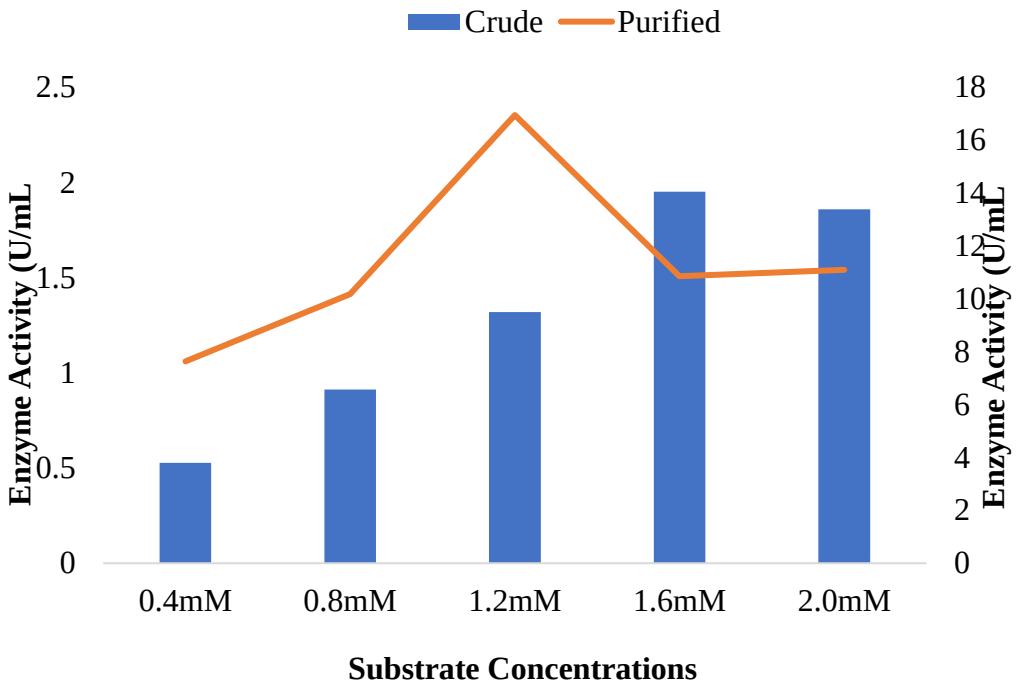


Figure 4.59: Effect of substrate concentration on activity of crude and purified L–asparaginase produced from *Amycolatopsis japonica.*



4.60: Effect of different substrate concentrations on activity of crude and purified L–asparaginase produced from Sp*hingobium yanoikuyae*

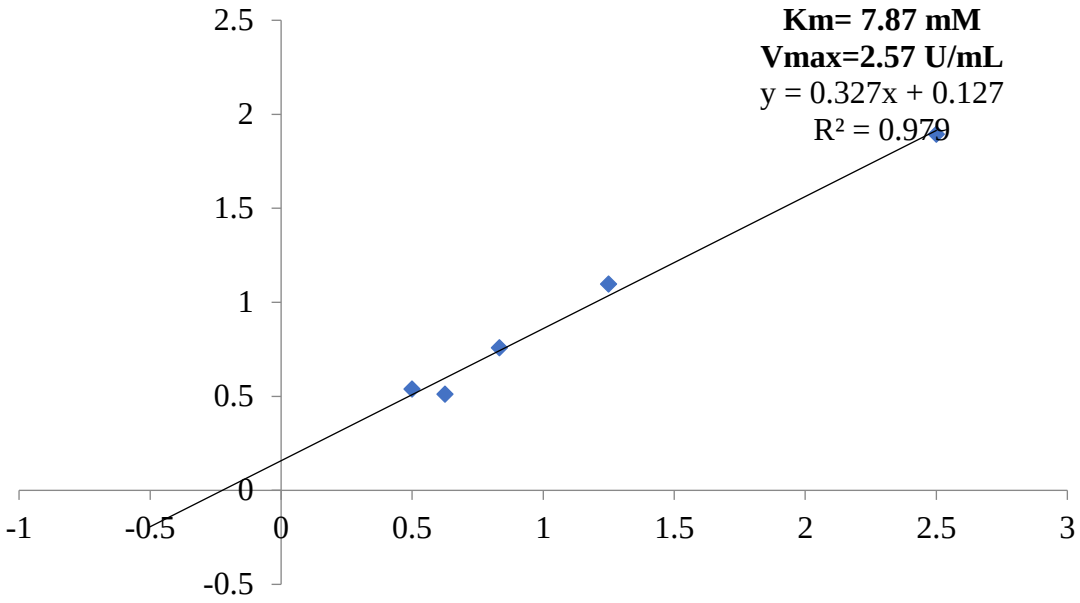


Figure 4.61: Line weaver plot for the reaction kinetics of crude L-asparaginase from *Amycolatopsis japonica.*

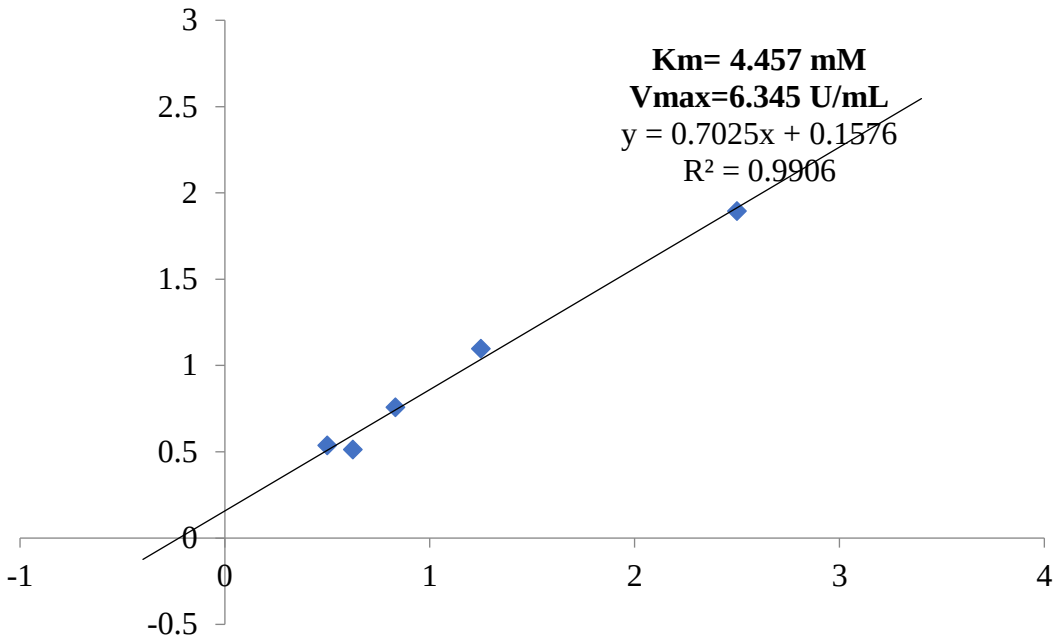


Figure 4.62: Line weaver plot for the reaction kinetics of crude L-asparaginase from *Sphingobium yanoikuyae*

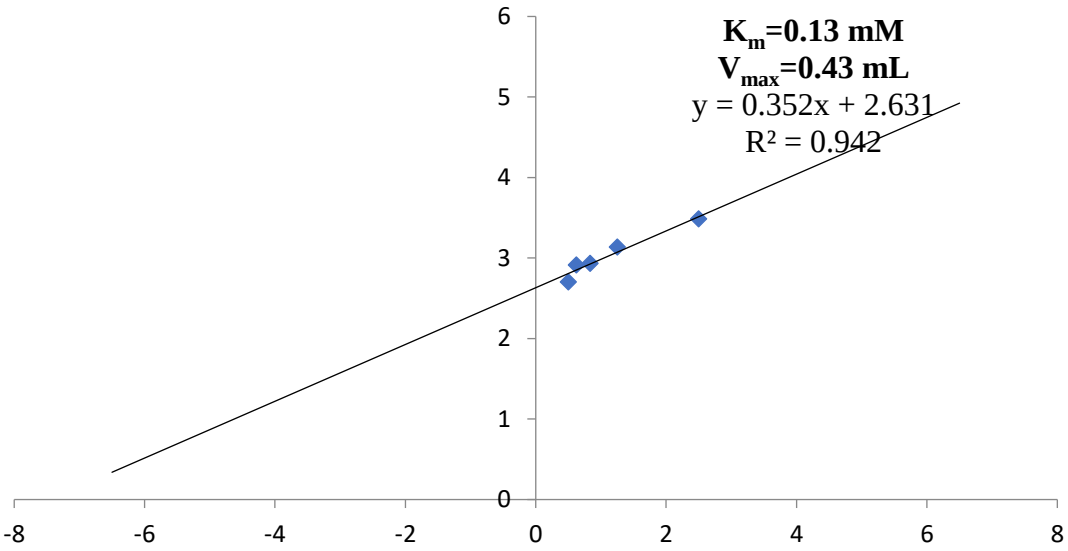


Figure 4.63: Line weaver plot for the reaction kinetics of purified L-asparaginase from *Amycolatopsis japonica*

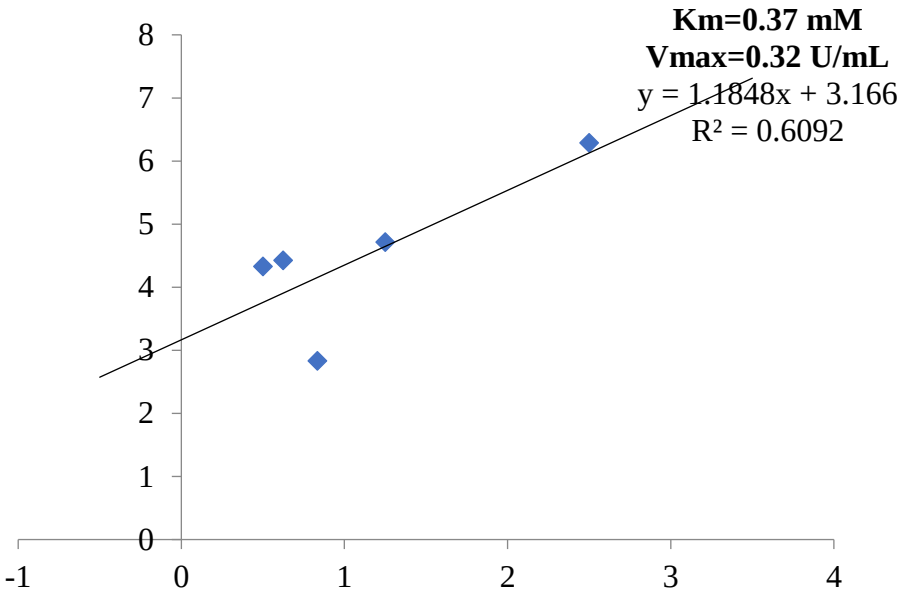


Figure 4.64: Line weaver plot for the reaction kinetics of purified L-asparaginase from *Sphingobium yanoikuyae*

4.6.10. FourierTransformed Infra-red (FTIR) Spectroscopy of L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae*

Studying the functional groups present in L-asparaginase produced from *Amycolatopsis japonica* Table 4.10 to 4.13 and comparing it with different ammonium sulphate fractions of partially purified enzyme after column chromatography purification, it was observed that the crude enzyme contain 1" 2" amine amide, carboxylic acid, Alkanes, 1" Amine, Aromatic and Aliphatic amine group. Compared to this, the 0 – 40% fraction contained Carboxylic acid, Aromatic, Aliphatic amine and Akyl halides group. There was no 1" 2" Amine, amide present in the crude. 40 – 80% fraction contained 1" 2" Amine, amide, Carboxylic acid, Aromatic, Aliphatic amine and Akyl halides group. In 80 – 100% fraction, aliphatic amide, carboxylic acid, aromatic groups was detected as compared to the crude and the other fractions was noticed. It was observed that in all the fractions tested (both the crude and the fractions tested), aliphatic amine group was present.

The result of functional group present in L-asparaginase produced from *Sphingobium yanoikuyae* is as recorded in Table 4.12 to 4.14. It was observed that the crude enzyme contained 1" 2" Amine, amide, Carboxylic acid, Aromatic, Aliphatic amine and Akyl halides. The 0 – 40 % and 40 – 80% ammonium sulphate fractions revealed the presence of Carboxylic acid, Alkene, Aromatic, Aliphatic amine and Akyl halides. No 1" 2" Amine, amide was detected in these fractions compared to the crude. Aliphatic amine, Akyl halides and Aromatic were present in all the samples tested.

Table 4.9: Fourier Transformed Infra-red (FTIR) Spectroscopy of crude L-asparaginase from *Amycolatopsis japonica*

	FREQUENCY	BOND	FUNCTIONAL GROUP
1.	3380.1	N –H Stretch	1” 2” Amine, amide
2.	3116.2	O-H Stretch	Carboxylic acid
3.	2962.0	C – H Stretch	Alkanes
4.	1677.5	N-H bend	1” Amine
5.	1638.6	C –C stretch	Aromatic
6.	1309.6	C-N Stretch	Aromatic
7.	1233.9	C – N Stretch	Aliphatic amine

Table 4.10: Fourier Transformed Infra-red (FTIR) Spectroscopy of partially purified L-asparaginase from *Amycolatopsis japonica* (0 – 40 %) Ammonium sulphate fraction.

	FREQUENCY	BOND	FUNCTIONAL GROUP
1.	3227.6	OH Stretch	Carboxylic acid
2.	1631.9	C=C Stretch	Aromatics
3.	1294.9	C –H Wag (CH_2X)	Akyl halides
4.	1112.4	C – N Stetch	Aliphatic amine
5.	617.9	C- Cl	Akyl halides

Table 4. 11: Fourier Transformed Infra-red (FTIR) Spectroscopy of partially purified L-asparaginase from *Amycolatopsis japonica* (40 – 80 %) Ammonium sulphate fraction.

	FREQUENCY	BOND	FUNCTIONAL GROUP
1.	3410.9	N –H Stretch	1” 2” Amine, amide
2.	3225.6	O-H Stretch	Carboxylic acid
3.	1638.0	C – C Stretch	Aromatic
4.	1486.1	C – C Stretch	Aromatic
5.	1115.8	C –N Stretch	Aliphatic amine
6.	619.1	C – C l stretch	Akyl halides

Table 4.12: Fourier Transformed Infra-red (FTIR) Spectroscopy of partially purified L-asparaginase from *Amycolatopsis japonica* (80 -100 %) Ammonium sulphate fraction.

	FREQUENCY	BOND	FUNCTIONAL GROUP
1.	3133.8	O - H Stretch	Carboxylic acid
2.	1639.4	C –C Stretch	Aromatic
3.	1113.9	C – N Stretsh	Aliphatic amines
4.	619.0	C- H bending	Alkanes

Table 4.13: Fourier Transformed Infra-red (FTIR) Spectroscopy of crude L-asparaginase from *Sphingobium yanoikuye.*

	FREQUENCY	BOND	FUNCTIONAL GROUP
1	3383.5	N-H Stretch	1” 2” Amine, amide
2	3120.6	O-H Stretch	Carboxylic acid
3.	1638.2	C=C Stretch	Aromatics
4.	1070.5	C-N Stretch	Aliphatic Amide
5.	530	C- Br stretch	Akyl halides

Table 4.14: Fourier Transformed Infra-red (FTIR) Spectroscopy of partially purified L-asparaginase from *Sphnigobium yanoikuy*e (0 -40 %) Ammonium sulphate fraction.

	FREQUENCY	BOND	FUNCTIONAL GROUP
1.	3228.7	OH Stretch	Carboxylic acid
2.	3006.1	C – H Stretch	Alkene
3.	1632.3	C – C Stretch	Aromatic
4.	1111.4	C – N Stretch	Aliphatic amine
5.	619.0	C – Cl Stretch	Akyl halides

Table 4.15: Fourier Transformed Infra-red (FTIR) Spectroscopy of partially purified L-asparaginase from *Sphingobium yanoikuy*e(40 -80%) Ammonium sulphate fraction.

	FREQUENCY	BOND	FUNCTIONAL GROUP
1.	3227.0	OH Stretch	Carboxylic acid
2.	3006.0	C – H Stretch	Alkene
3.	1631.2	C – C Stretch	Aromatic
4.	1295.5	C –H Wag (CH_2X)	Akyl halides
5.	1115.8	C – N Stretch	Aliphatic amine
6.	617.5	C – Cl Stretch	Akyl halide

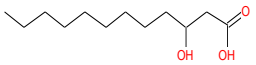
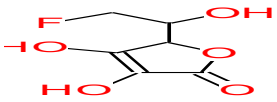
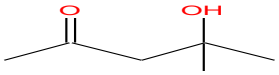
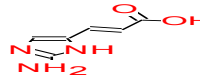
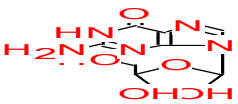
4.6.11. Metabolite profiling of L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuye*

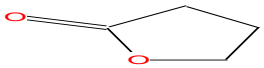
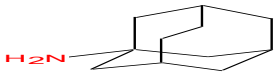
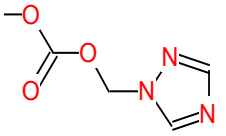
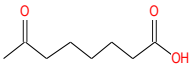
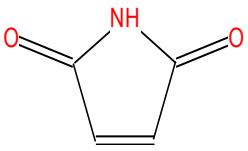
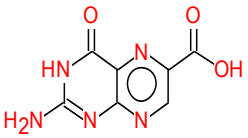
The chemical composition of crude L-asparaginase from *A. Japonica* and *S. Yanoikuye* was investigated using GC-MS analysis (Table 16 and 17). On

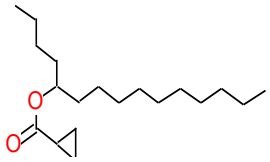
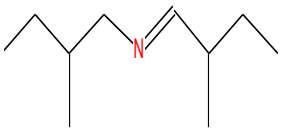
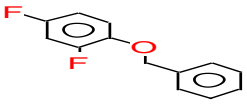
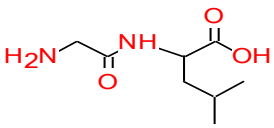
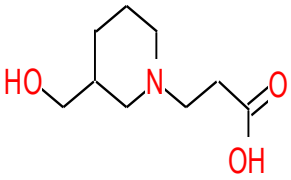
comparison of the mass spectra of each peak with that spectrum stored on the National Insttute of Standard and Technology (NIST) library, several compounds were identified by retention time, molecular weight and molecular formula which range from straight chain hydrocarbons or alcohols, to ammonia derivative and series of amino acids residues. They were Dodecanoic acid, 3-hydroxy-, Furan-2-one, 3,4-dihydroxy-5-[1-hydroxy-2-fluoroethyl]-, 2-Pentanone, 4-hydroxy-4-methyl-, l-Gala-l-ido-octose, Imidazole, 2-amino-5-[(2-carboxy)vinyl]-, Guanosine, Butyrolactone, Tricyclo[4.3.1.1(3,8)]undecan-1-amine, Carbonic acid, (ethyl)(1,2,4-triazol-1-ylmethyl) diester, Octanoic acid, 7-oxo-, 1H-Pyrrole-2,5-dione, Pterine-6-carboxylic acid, 5-Cyclopropylcarbonyloxypentadecane, 1-Butanamine, 2-methyl-N-(2-methylbutylidene)-, 2,4-Difluorobenzene1-benzyloxy-, DL-Leucine, N-glycyl-. 1-Pyridinepropanoic acid, hexahydro-3-(hydroxymethyl, Glycylsarcosine, 2-Pyrrolidinone, Imidazole, 4-methyl-5-[2-methyl-2-propenyl]-, Piperidine-2,5-dione, 3-Trifluoroacetoxypentadecane, 9-Oxabicyclo[3.3.1]nonane-2,6-diol, 2,5-Pyrrolidinedione, Pyrimidine-2,4,6-trione, 1-cyclohexyl-5-[(2-piperazin-1-yl-ethylamino)methylene]-, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, 1-(4-Amino-furazan-3-yl)-5-(4-methyl-piperazin-1-ylmethyl)-1H-[1,2,3]triazole-4-carboxylic acid ethyl ester, Arginine, 2,6-Naphthalenediol, 1,5-bis[[3-(4-methylpiperazino)propylimino]methyl]-, Uric acid, Acetate, [3-(acetyloxy)-4,5-dihydro-5-isoxazolyl]methyl, d-Gala-l-ido-octonic amide, Dec-9-en-6-oxo-1-ylamide, Mannosamine, Deoxyspergualin, 2,7-Dioxatricyclo[4.4.0.0(3,8)]decan-4-amine, stereoisomer, 1-Methyl-4-[nitromethyl]-4-piperidinol, Paromomycin, Butanoic acid, 4,4'-dithiobis[2-amino-, (R*,R*)-(±)-, 3-[N-[2-Diethylaminoethyl]-1 cyclopentenylamino]propionitrile, DL-Norleucine, Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]-, 1(2H)-Naphthalenone, 2-amino-2,3-dihydro-, Pyrrolizin-1,7-dione-6-carboxylic acid, methyl(ester), 10-Heptadecen-8-ynoic acid, methyl ester, (E)-, Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, Cyclopentanone, 2-(2-octenyl)-, L-Aspartic acid, N-glycyl-, 2-Myristynoyl pantetheine, Actinomycin C, 3,7-Diazabicyclo[3.3.1]nonane, 9,9-dimethyl-, n-Hexadecanoic acid, n-Propyl 9-tetradecenoate, Phen-1,4-diol, 2,3-dimethyl-5-trifluoromethyl-, 9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3β,5Z,7E)-, Methotrexate, Cystine, Pentanol, 5-amino-, L-Serine, O-(phenylmethyl)-, dl-Allo-cystathionine, Azetidin-2-one 3,3-

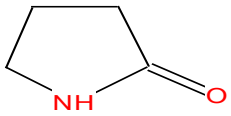
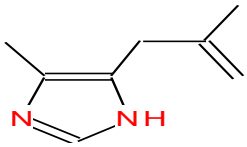
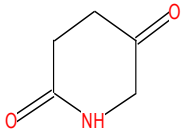
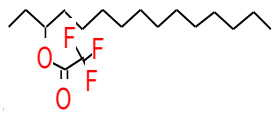
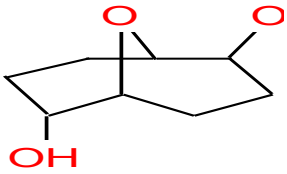
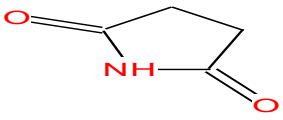
dimethyl-4-(1-aminoethyl, Niacinamide, Folic acid, Glycyl-D-asparagine, Gentamicin a, Oleic Acid.

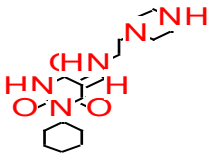
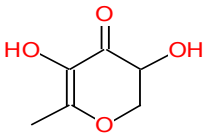
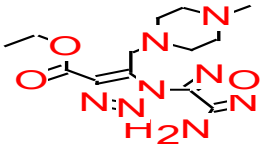
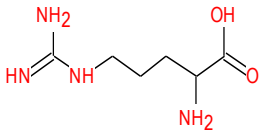
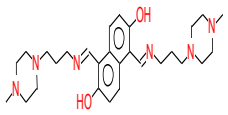
Table 4.16: Metabolites profiling of L-asparaginase produced from *Amycolatopsis japonica*

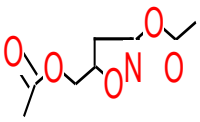
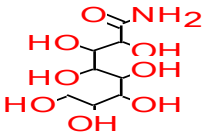
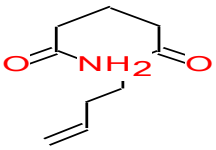
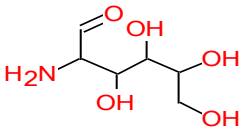
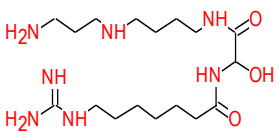
S/no	Compound	Synonyms	Chemical Formular	Structure and Molecular weight
1	Dodecanoic acid, 3-hydroxy-	1.β-Hydroxydodecanoic acid 2.β-Hydroxylauric acid 3.3-Hydroxydodecanoic acid	$C_{12}H_{24}O_3$	 216g/mol
2	Furan-2-one, 3,4-dihydroxy-5-[1-hydroxy-2-fluoroethyl]-	No Synonyms	$C_6H_7FO_5$	 178g/mol
3	2-Pentanone, 4-hydroxy-4-methyl-	1.Acetonyldimethylcarbinol 2.Diacetone alcohol	$C_6H_{12}O_2$	 116g/mol
4	l-Gala-l-ido-octose	No Synonyms	$C_8H_{16}O_8$	 240g/mol
5	Imidazole, 2-amino-5-[(2-carboxy)vinyl]-	No Synonyms	$C_6H_7N_3O_2$	 153g/mol
6	Guanosine	1.Guanine, 9β-d-ribofuranosyl- 2.β-d-Ribofuranoside, guanine-9 3.Guanozin 4.Guo 5.Inosine, 2-amino-	$C_{10}H_{13}N_5O_5$	 283g/mol

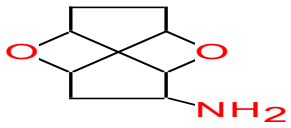
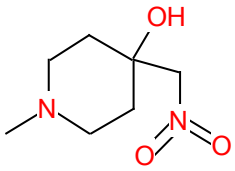
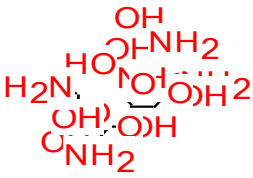
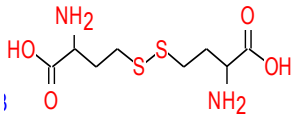
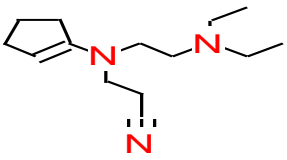
		6.Vernine		
7	Butyrolactone	1.2(3H)-Furanone, dihydro- 2.γ-Butyrolactone	$C_4H_6O_2$	 86g/mol
8	Tricyclo[4.3.1.1(3,8)]undecan-1-amine	No Synonyms	$C_{11}H_{19}N$	 165g/mol
9	Carbonic acid, (ethyl)(1,2,4-triazol-1-ylmethyl) diester		$C_6H_9N_3O_3$	 171g/mol
10	Octanoic acid, 7-oxo-	7-Oxooctanoic acid	$C_8H_{14}O_3$	 158g/mol
11	1H-Pyrrole-2,5-dione	1.Maleimide 2.Pyrrole-2,5-dione 3.3-Pyrroline-2,5-dione 4.Maleinimide 5.Maleic imide	$C_4H_3NO_2$	 97g/mol
12	Pterine-6-carboxylic acid	1.2-Amino-4-hydroxypteridine-6-carboxylic acid.	$C_7H_5N_5O_3$	 207g/mol

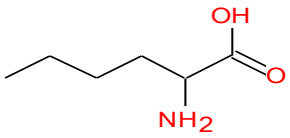
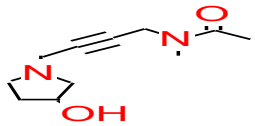
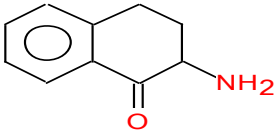
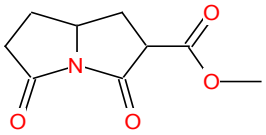
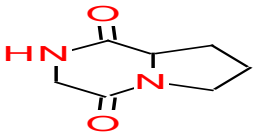
13	5-Cyclopropylcarbonyloxypentadecane	No Synonyms	$C_{19}H_{36}O_2$	 296g/mol
14	1-Butanamine, 2-methyl-N-(2-methylbutylidene)-	1. N-(2-Methylbutylidene)-2-methylbutylamine 2. 2-Methyl-N-[(E)-2-methylbutylidene]-1-butanamine #	$C_{10}H_{21}N$	 155g/mol
15	2,4-Difluorobenzene1-benzyloxy-	1. 1-(Benzyloxy)-2,4-ifluorobenzene	$C_{13}H_{10}F_2O$	 220g/mol
16	DL-Leucine, N-glycyl-	1.N-(Aminoacetyl)leucine	$C_8H_{16}N_2O_3$	 188g/mol
17	1-Pyridinepropanoic acid, hexahydro-3-(hydroxymethyl)-	No Synonyms	$C_9H_{17}NO_3$	 187g/mol
18	Glycylsarcosine	Synonyms: 1.Glycine, N-glycyl-N-methyl- 2.[(Aminoacetyl)(methyl)amino]acetic acid	$C_5H_{10}N_2O_3$	 146g/mol

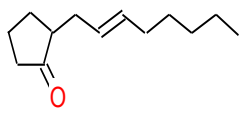
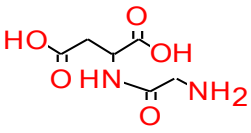
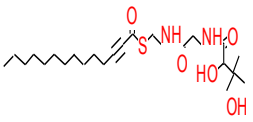
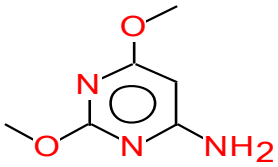
19	2-Pyrrolidinone	1.γ-Aminobutyrolactam 2.α-Pyrrolidinone 3.α-Pyrrolidone 4.γ-Aminobutyric lactam 5.γ-Butyrolactam	C_4H_7NO	 85g/mol
20	Imidazole, 4-methyl-5-[2-methyl-2-propenyl]-		$C_8H_{12}N_2$	 136g/mol
21	Piperidine-2,5-dione	1.2,5-Piperidinedione	$C_5H_7NO_2$	 113g/mol
22	3-Trifluoroacetoxypentadecane		$C_{17}H_{31}F_3O_2$	 324g/mol
23	9-Oxabicyclo[3.3.1]nonane-2,6-diol	No Synonyms	$C_8H_{14}O_3$	 158g/mol
24	2,5-Pyrrolidinedione	1.Succinimide 2.Butanimide 3.Succinic acid imide 4.Succinic imide	$C_4H_5NO_2$	 99g/mol

25	Pyrimidine-2,4,6-trione, 1-cyclohexyl-5-[(2-piperazin-1-yl-ethylamino)methylene]-	No Synonyms	$C_{17}H_{27}N_5O_3$	 349g/mol
26	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	.3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	$C_6H_8O_4$	 144g/mol
27	1-(4-Amino-furazan-3-yl)-5-(4-methyl-piperazin-1-ylmethyl)-1H-[1,2,3]triazole-4-carboxylic acid ethyl ester	No Synonyms	$C_{13}H_{20}N_8O_3$	 336 g/mol
28	Arginine	1.L-Arginine 2.Arginine, L-	$C_6H_{14}N_4O_2$	 174g/mol
29	2,6-Naphthalenediol, 1,5-bis[[3-(4-methylpiperazino) propylimino]methyl]-	No Synonyms	$C_{28}H_{42}N_6O_2$	 494g/mol

30	Uric acid	1.1H-Purine-2,6,8(3H)-trione, 7,9-dihydro- 2.Lithic acid 3.Purine-2,6,8(1H,3H,9H)-trione 4.1H-Purine-2,6,8-triol	$C_5H_4N_4O_3$	 168g/mol
31	Acetate, [3-(acetyloxy)-4,5-dihydro-5-isoxazolyl]methyl	No Synonyms	$C_8H_{11}NO_5$	 201g/mol
32	d-Gala-l-ido-octonic amide	No Synonyms	$C_8H_{17}NO_8$	 255g/mol
33	Dec-9-en-6-oxo-1-ylamide	No Synonyms	$C_{10}H_{17}NO_2$	 183g/mol
34	Mannosamine	No Synonyms	$C_6H_{13}NO_5$	 179g/mol
35	Deoxyspergualin	No Synonyms	$C_{17}H_{37}N_7O_3$	 387g/mol

36	2,7-Dioxatricyclo[4.4.0.0(3,8)]decan-4-amine, stereoisomer	No Synonyms	$C_8H_{13}NO_2$	 155g/mol
37	1-Methyl-4-[nitromethyl]-4-piperidinol	No Synonyms	$C_7H_{14}N_2O_3$	 174g/mol
38	D-Streptamine, O-2-amino-2-deoxy-α-D-glucopyranosyl-(14)-O-[O-2,6-diamino-2,6-dideoxy-β-L-idopyranosyl-(13)-β-D-ribofuranosyl-(15)]-2-deoxy-	.Paromomycin i 2.Amminosidin 3.Catenulin 4.Crestomycin	$C_{23}H_{45}N_5O_{14}$	 615g/mol
39	Butanoic acid, 4,4'-dithiobis[2-amino-, (R*,R*)-(±)-		C8H16N2O4S2	 268g/mol
40	3-[N-[2-Diethylaminoethyl]-1-cyclopentenylamino]propionitrile		$C_{14}H_{25}N_3$	 235g/mol

41	DL-Norleucine	1.Norleucine, DL- 2.(±)-Norleucine 3.DL-α-Aminocaproic acid	$C_6H_{13}NO_2$	 131g/mol
42	Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]-	N-[4-(3-Hydroxy-1-pyrrolidinyl)-2-butynyl]-N-methylacetamide #	$C_{11}H_{18}N_2O_2$	 210g/mol
43	1(2H)-Naphthalenone, 2-amino-2,3-dihydro-	1.2-Amino-3,4-dihydro-1(2H)-naphthalenone	$C_{10}H_{11}NO$	 161g/mol
44	Pyrrolizin-1,7-dione-6-carboxylic acid, methyl(ester)	Methyl 3,5-dioxohexahydro-1H-pyrrolizine	$C_9H_{11}NO_4$	 197g/mol
45	10-Heptadecen-8-ynoic acid, methyl ester, (E)-	Methyl (10E)-10-heptadecen-8-ynoate	$C_{18}H_{30}O_2$	 278g/mol
46	Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione	Pyrrolidino[1,2-a]piperazine-3,6-dione	$C_7H_{10}N_2O_2$	 154g/mol

47	Cyclopentanone, 2-(2-octenyl)-	No Synonyms	$C_{13}H_{22}O$	 194g/mol
48	L-Aspartic acid, N-glycyl-	.N-(Aminoacetyl)aspartic acid	$C_6H_{10}N_2O_5$	 190g/mol
49	2-Myristynoyl pantetheine		$C_{25}H_{44}N_2O_5S$	 484g/mol
50	2,6-Dimethoxy-4-aminopyrimidine	1.4-Pyrimidinamine, 2,6-dimethoxy- 2.Pyrimidine, 4-amino-2,6-dimethoxy- 3.4-Amino-2,6-dimethoxypyrimi	$C_6H_9N_3O_2$	 155g/mol
51	Actinomycin	1.Actinomycin D, 2A-D-alloisoleucine- 2.Actinomycin VI 3.Actinomycin D, 2(sup A)-D-alloisoleucine- 4.NSC 87221	$C_{63}H_{88}N_{12}O_{16}$	1268g/mol

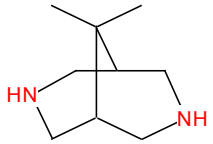
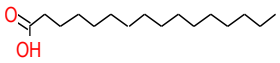
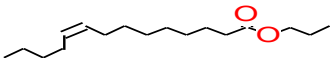
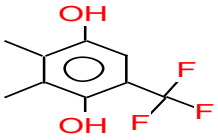
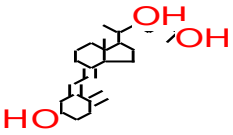
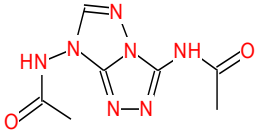
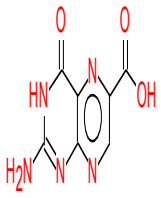
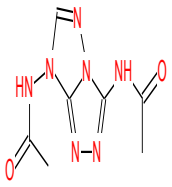
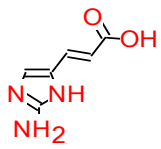
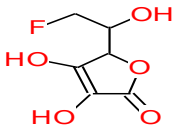
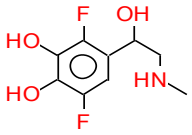
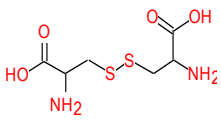
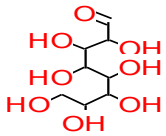
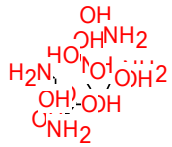
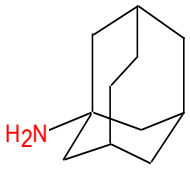
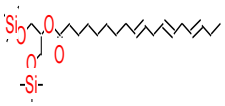
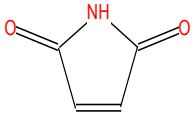
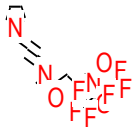
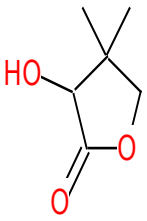
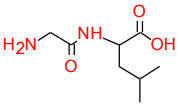
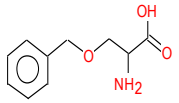
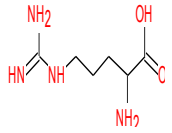
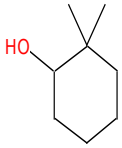
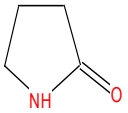
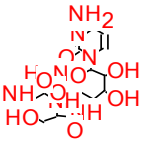
52	3,7-Diazabicyclo[3.3.1]nonane, 9,9-dimethyl-		$C_9H_{18}N_2$	 154g/mol
53	n-Hexadecanoic acid	1.Hexadecanoic acid 2.Palmitic acid	$C_{16}H_{32}O_2$	 256g/mol
54	n-Propyl 9-tetradecenoate	No Synonyms	$C_{17}H_{32}O_2$	 268g/mol
55	Phen-1,4-diol, 2,3-dimethyl-5-trifluoromethyl-		$C_9H_9F_3O_2$	 206g/mol
56	9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3β,5Z,7E)-	1.24,25-Dihydroxycholecalciferol 2.24,25-Dihydroxyvitamin D3	$C_{27}H_{44}O_3$	 416g/mol
57	Methotrexate	1.L-Glutamic acid, N-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-	$C_{20}H_{22}N_8O_5$	 454g/mol
58	3,7-Diacetamido-7H-s-triazolo[5,1-c]-s-triazole	No Synonyms	$C_7H_9N_7O_2$	 223g/mol

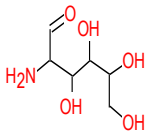
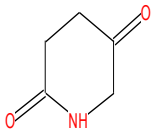
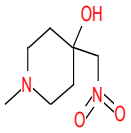
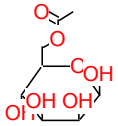
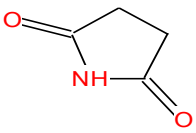
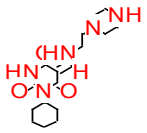
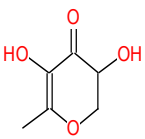
Table 4.16: Metabolites profiling of L- asparaginase produced from *Sphingobium yanoikuye*

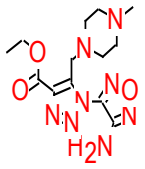
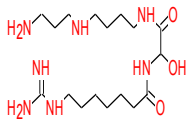
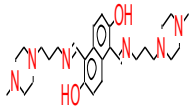
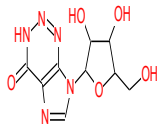
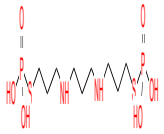
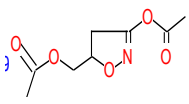
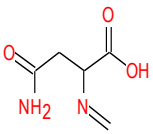
S/no	Name	Synonyms	Chemical Formular	Structure/mw
1	Pterin-6-carboxylic acid	1. 2-Amino-4-hydroxypteridine-6-carboxylic acid 2. 2-Amino-4-oxo-3,4-dihydro-6-pteridinecarboxylic acid	$C_7H_5N_5O_3$	 207g/mol
2	3,7-Diacetamido-7H-s-triazolo[5,1-c]-s-triazole	No Synonyms	$C_7H_9N_7O_2$	 223g/mol
3	Imidazole, 2-amino-5-[(2-carboxy)vinyl]-	No Synonyms	$C_6H_7N_3O_2$	 153g/mol

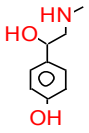
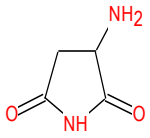
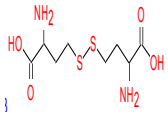
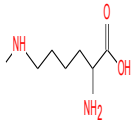
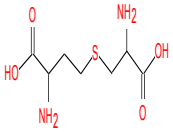
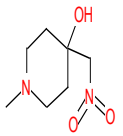
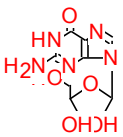
4	Furan-2-one, 3,4-dihydroxy-5-[1-hydroxy-2-fluoroethyl]-	No Synonyms	$C_6H_7FO_5$	 178g/mol
5	Benzeneethanamine, 2,5-difluoro-β,3,4-trihydroxy-N-methyl-	1.3,6-Difluoro-4-[1-hydroxy-2-(methylamino)ethyl]-1,2-benzenediol	$C_9H_{11}F_2NO_3$	 219g/mol
6	Cystine	1.l-Cystine 2.β,β'-Diamino-β,β'-dicarboxydiethyl disulfide 3.β,β'-Dithiodialanine 4.Alanine, 3,3'-dithiobis-	$C_6H_{12}N_2O_4S_2$	 240g/mol
7	l-Gala-l-ido-octose	No Synonyms	$C_8H_{16}O_8$	 240g/mol
8	D-Streptamine, O-2-amino-2-deoxy-α-D-glucopyranosyl-(14)-O-[O-2,6-diamino-2,6-dideoxy-β-L-idopyranosyl-(13)-β-D-ribofuranosyl-(15)]-2-deoxy-	<u>Synonyms:</u> 1.Paromomycin i 2.Amminosidin 3.Catenulin 4.Crestomycin	$C_{23}H_{45}N_5O_{14}$	 615g/mol
9	Pentanol, 5-amino-	<u>Synonyms:</u> 1.5-Amino-1-pentanol	$C_5H_{13}NO$	 103g/mol

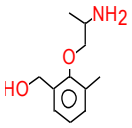
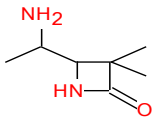
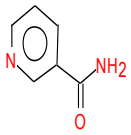
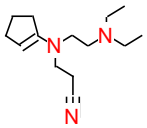
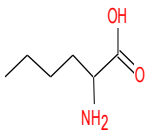
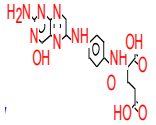
		2. 5-Aminopentanol-1		
10	: Tricyclo[4.3.1.1(3,8)]undecan-1-amine	No Synonyms	$C_{11}H_{19}N$	 165g/mol
11	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)-	1.2-[(Trimethylsilyl)oxy]-1-([(trimethylsilyl)oxy]methyl)ethyl (9E,12E,15E)-9,12	$C_{27}H_{52}O_4Si_2$	 496g/mol
12	1H-Pyrrole-2,5-dione	1.Maleimide 2.Pyrrole-2,5-dione 3.3-Pyrroline-2,5-dione	$C_4H_3NO_2$	 97g/mol
13	4-Aminobutyramide, N-methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-N',N'-bis(trifluoroacetyl)-	No Synonyms	$C_{17}H_{21}F_6N_3O_3$	 429g/mol
14	Pantolactone	1.2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (R)- 2.2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, D-(-)-	$C_6H_{10}O_3$	 130g/mol
15	DL-Leucine, N-glycyl-	1.N-(Aminoacetyl)leucine	: $C_8H_{16}N_2O_3$	 188g/mol

16	L-Serine, O-(phenylmethyl)-	1.O-Benzyl-L-serine 2.Benzyl ester of L-Serine	$C_{10}H_{13}NO_3$	 195g/mol
17	Arginine	1.L-Arginine 2.Arginine, L- 3.L-(+)-Arginine 4.Norvaline, 5-[(aminoiminomethyl) amino]-	$C_6H_{14}N_4O_2$	 174 g/mol
18	Cyclohexanol, 2,2-dimethyl-	Dimethylcyclohexanol	$C_8H_{16}O$	 128 g /mol
19	2-Pyrrolidinone	1.γ-Aminobutyrolactam 2.α-Pyrrolidinone 3.α-Pyrrolidone	C_4H_7NO	 85g/mol
20	E-7-Tetradecenol	no synonyms	$C_{14}H_{28}O$	 212g/mol
21	Glucopyranuronamide, 1-(4-amino-2-oxo-1(2H)-pyrimidinyl)-1,4-dideoxy-4-(D-2-(2-(methylamino)acetamido)hydracrylamido)-, β-D-	1.Antibiotic 21544 2.Aspiculamycin 3.Asteromycin 4.1-(4-Deoxy-4-(sarcosyl-D-seryl)amino-β-D-glucopyranuronamide)cytosine	$C_{16}H_{25}N_7O_8$	 443g/mol

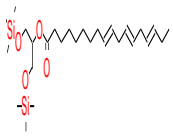
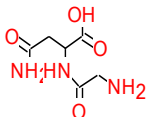
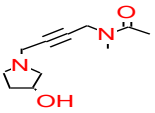
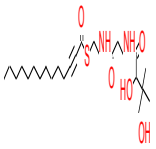
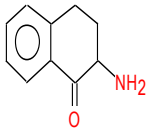
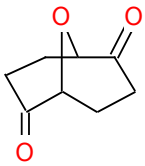
		4.Quingfengmycin		
22	Mannosamine	no synonyms	$C_6H_{13}NO_5$	 179g/mol
23	Piperidine-2,5-dione	1.2,5-Piperidinedione	$C_5H_7NO_2$	 131g/mol
24	1-Methyl-4-[nitromethyl]-4-piperidinol	no synonyms	$C_7H_{14}N_2O_3$	 174g/mol
25	6-Acetyl-β-d-mannose	no synonyms.	$C_8H_{14}O_7$	 222g/mol
26	2,5-Pyrrolidinedione	1.Succinimide 2.Butanimide 3.Succinic acid imide	$C_4H_5NO_2$	 99g/mol
27	Pyrimidine-2,4,6-trione, 1-cyclohexyl-5-[(2-piperazin-1-yl-ethylamino)methylene]-	no synonyms	$C_{17}H_{27}N_5O_3$	 349g/mol
28	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	no synonyms	$C_6H_8O_4$	 144g/mol

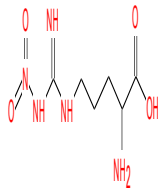
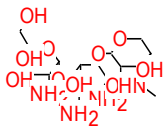
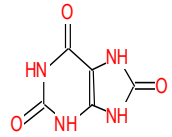
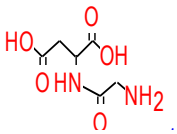
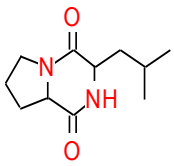
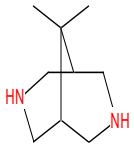
29	1-(4-Amino-furazan-3-yl)-5-(4-methyl-piperazin-1-ylmethyl)-1H-[1,2,3]triazole-4-carboxylic acid ethyl ester	no synonyms	$C_{13}H_{20}N_8O_3$	 336g/mol
30	Deoxyspergualin	no synonyms.	: $C_{17}H_{37}N_7O_3$	 387g/mol
31	2,6-Naphthalenediol, 1,5-bis[[3-(4-methylpiperazino)propylimino]methyl]-	no synonyms.	$C_{28}H_{42}N_6O_2$	 494g/mol
32	7-[β-d-Ribofuranosyl]imidazo[4,5-d][1,2,3]-triazin-4-one (2-azainosine)	no synonyms.	$C_9H_{11}N_5O_5$	 269g/mol
33	Phosphorothioic acid, S-ester with trimethylenediiminodipropanethiol (2:1)	1.N,N'-Trimethylenebis(sodium S-2-aminoethylhydrogen phosphorothioate) 2.N,N'-Trimethylenebis[s-3-aminopropyl-phosphorothioic acid	$C_9H_{24}N_2O_6P_2S_2$	 382g/mol
34	Acetate, [3-(acetyloxy)-4,5-dihydro-5-isoxazolyl]methyl	no synonyms.	$C_8H_{11}NO_5$	 201g/mol
35	Methylene asparagine	no synonyms.	$C_5H_8N_2O_3$	 144g/mol

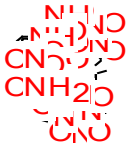
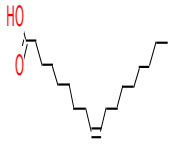
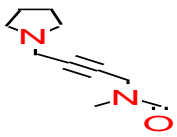
36	Benzyl alcohol, p-hydroxy-α-[(methylamino)methyl]-	1.β-Methylamino-α-(4-hydroxyphenyl)ethyl alcohol	$C_9H_{13}NO_2$	 167g/mol
37	Tetrahydropyrrole-3-amino-2,5-dione	no synonyms.	$C_4H_6N_2O_2$	 114g/mol
38	Butanoic acid, 4,4'-dithiobis[2-amino-, [S-(R*,R*)]-	no synonyms.	$C_8H_{16}N_2O_4S_2$	 268g/mol
39	N(Epsilon)-methyl-l-lysine	no synonyms.	$C_7H_{16}N_2O_2$	 160g/mol
40	dl-Allo-cystathionine	no synonyms.	$C_7H_{14}N_2O_4S$	 222g/mol
41	1-Methyl-4-[nitromethyl]-4-piperidinol	no synonyms.	$C_7H_{14}N_2O_3$	 174g/mol
42	Guanosine	1.Guanine, 9β-d-ribofuranosyl- 2.β-d-Ribofuranoside, guanine-9 3.Guanozin 4.Guo	$C_{10}H_{13}N_5O_5$	 283g/mol

43	Benzenemethanol, 2-(2-aminopropoxy)-3-methyl-	1.[2-(2-Aminopropoxy)-3-methylphenyl]methanol #	$C_{11}H_{17}NO_2$	 195g/mol
43	Azetidin-2-one 3,3-dimethyl-4-(1-aminoethyl)-	no synonyms.	$C_7H_{14}N_2O$	 195g/mol
44	Niacinamide	1.3-Pyridinecarboxamide 2.Nicotinamide 3.β-Pyridinecarboxamide 4.Aminicotin	$C_6H_6N_2O$	 122g/mol
45	3-[N-[2-Diethylaminoethyl]-1-cyclopentenylamino]propionitrile	no synonyms	$C_{14}H_{25}N_3$	 235g/mol
46	DL-Norleucine	1.Norleucine, DL- 2.(±)-Norleucine 3.DL-α-Aminocaproic acid 4.2-Aminohexanoic acid	$C_6H_{13}NO_2$	 131g/mol
47	Folic acid	1.L-Glutamic acid, N-[4-[[(2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]- 2.Acifolic	$C_{19}H_{19}N_7O_6$	 441g/mol

3.Cytofol

48	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)-	1.2-[(Trimethylsilyl)oxy] -1-([(trimethylsilyl)oxy] methyl)ethyl (9E,12E,15E)-9,12,15-octadecatrienoate	$C_{27}H_{52}O_4Si_2$	 496g/mol
49	Glycyl-D-asparagine	no synonyms	$C_6H_{11}N_3O_4$	 189g/mol
50	Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]-	1.N-[4-(3-Hydroxy-1-pyrrolidinyl)-2-butynyl]-N-methylacetamide	$C_{11}H_{18}N_2O_2$	 210g/mol
51	2-Myristynoyl pantetheine	no synonyms	$C_{25}H_{44}N_2O_5S$	 484g/mol
52	1(2H)-Naphthalenone, 2-amino-2,3-dihydro-	1.2-Amino-3,4-dihydro-1(2H)-naphthalenone	$C_{10}H_{11}NO$	 161g/mol
53	9-Oxa-bicyclo[3.3.1]nonane-2,6-dione	no synonyms.	$C_8H_{10}O_3$	 154g/mol

54	Nitro-L-arginine	1.N.w-nitro-L-arginine 2.ω-Nitro-L-arginine 3.w-Nitro-L-arginine	$C_6H_{13}N_5O_4$	 219g/mol
55	Gentamicin a	1.4,6-Diamino-3-([3-deoxy-3-(methylamino)pentopyranosyl]oxy)-2-hydroxycy	$C_{18}H_{36}N_4O_{10}$	 468g/mol
56	Uric acid	1.1H-Purine-2,6,8(3H)-trione, 7,9-dihydro- 2.Lithic acid	$C_5H_4N_4O_3$	 168g/mol
57	L-Aspartic acid, N-glycyl-	1.N-(Aminoacetyl)aspartic acid	$C_6H_{10}N_2O_5$	 190g/mol
58	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	1. 3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	 210g/mol
59	3,7-Diazabicyclo[3.3.1]nonane, 9,9-dimethyl-	1.9,9-Dimethyl-3,7-diazabicyclo[3.3.1]nonane	$C_9H_{18}N_2$	 154g/mol

60	: Actinomycin C2	1.Actinomycin D, 2A-D-alloisoleucine- 2.Actinomycin VI 3.Actinomycin D, 2(sup A)-D-alloisoleucine	$C_{63}H_{88}N_{12}O_{16}$	 1268g/mol
61	Oleic Acid	1.9-Octadecenoic acid (Z)- 2.δ(Sup9)-cis-Oleic acid 3.cis-δ(Sup9)-Octadecenoic acid	$C_{18}H_{34}O_2$	 282g/mol
62	Formamide, N-methyl-N-4-[1-(pyrrolidinyl)-2-butynyl]-	1.Methyl[4-(1-pyrrolidinyl)-2-butynyl]formamide	$C_{10}H_{16}N_2O$	 180g/mol

4.7 Applications of L-asparaginase produced from *Amycolatopsis japonica and Sphingobium yanoikuyae.*

Different biological activities were studied on L-asparaginase produced by *Amycolatopsis japonica and Sphingobium yanoikuye* to check their industrial and medicinal usefulness. They were antibacterial activity, antifungal activity, antileishmanial activity, insecticidal activity, brine shrimps lethality assay, anticancer activity and acrylamide reduction potential.

4.7.1 Antibacterial activity of L-asparaginase produced from *Amycolatopsis japonica and Sphingobium yanoikuyae.*

The L-asparaginase from *Amycolatopsis japonica* showed very low antibacterial activity on all the test organisms. The highest inhibitory activity of 19.5% was noticed against *Bacillus subtilis* and the lowest inhibitory activity (0.15%) of this enzyme was observed on *Pseudomonas aeroginosa*. Antibacterial activity of 8.9% and 7.7% of the enzyme was observed against *Enterococcus faecalis* and *Escherichia coli respectively*. L- asparaginase from *Sphingobium yanoikuyae* showed the highest inhibitory activity of 9.9% against *Bacillus subtilis* and the lowest inhibitory activity of 1.1% and 1.3% against *Pseudomonas aeroginosa* and *Enterococcus faecalis* respectively. No inhibitory activity of L-asparaginase from *Sphingobium yanoikuye* was noticed against *Staphylococcus aureus*. The antibacterial activity profile of L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuye* is presented in (Table 4.17).

4.7.2 Antifungal activity of L-asparaginase produced from *Amycolatopsis japonica and Sphingobium yanoikuyae.*

The effect of the L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuye* on pathogenic fungi like *Candida albican, Fusarium lini, Candida globurata, Microsporium canis, Trichophyton rubrum* and *Aspergillus niger* were studied. The L-asparaginase from *Amycolatopsis japonica* showed highest growth inhibition of 2.0cm against *Aspergillus niger,* followed by 1.5cm growth inhibition against *Candida globurata*. No inhibitory activity of the enzyme was seen on *Trichophyton rubrum*. The enzyme showed insignificant growth inhibition activity of 0.5cm against *Candida albican* and *Fusarium lini*. 1.0cm growth inhibition was observed from the enzyme against *Microsporum canis*. The L-asparaginase from

Sphingobium yanoikuye showed 1.5cm growth inhibition against *Candida albican* and *Microsporum canis*. 1,0cm growth inhibition was noticed against *Candida globurata*. No antifungal activity of L-asparaginase from *Sphingobium yanoikuye* was observed against *Fusarium lini, Trichophyton rubrum*, and *Aspergillus niger* (Table 4.17)

Table 4.18: Antimicrobial Activity of L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuaye*.

	Antibacterial Activity (Growth Inhibition%)					Antifungal Activity (Growth Inhibition(cm)					
	Bacillus subtilis	*Enterococcus faecalis*	*Escherichia*	*Staphylococcus aureus*	*Pseudomona aeruginosa*	*Candida*	*Fusarium lini*	*Candida*	*Microsporum*	*Trichophyton*	*Aspergillus*
Amycolatopsis	19.5 ±0.1	8.9 ± 0.1	7.7 ± 0.1	-7.4 ± 0.1	-0.15 ± 0.001	0.5 ± 1	0.5 ± 1	1.5 ± 1	1.0 ± 1	0 ± 1	2.0 ± 0.1
Sphingobium	9.9 ± 0.1	1.3 ± 0.1	4.9 ± 0.1	-10 ± 0.1	1.1 ± 0.1	1.5 ± 1	0 ± 1	1.0 ± 1	1.5 ± 1	0 ± 1	0 ± 1
Control	0.222 ±0.001	0.52 ±0.01	0.93 ±0.01	-28.145 ± 0.1	-8.47 ± 0.1	5.0 ± 1	5.0 ± 1	5.0 ± 1	5.0 ± 1	5.0 ± 1	5.0 ± 1

4.7.3 Insecticidal activity of L-asparaginase produced from *Amycolatopsis japonica and Sphingobium yanoikuye.*

The ability of the L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuye* to cause the death of insects was studied on two rice insects (*Sytophillus spp* and *Ryzopertha spp*). From the result, L-asparaginase from *Amycolatopsis japonica* showed some degree of insecticidal activity against one of the insects. 40% insecticidal activity of the enzyme was recorded against *Sytophillus* species as 4 out of the 10 insects used were dead after 24 hours. and no effect of the enzyme was recorded on *Ryzopertha* species. L-asparaginase from *Sphingobium yanoikuye* showed 100% insecticidal activity against *Ryzopertha* spp. as death of all the insects used was recorded after 24 hours and no insecticidal activity was recorded against *Sytophillus* species (Table 4.18)

Table 4.18: Insecticidal potential of L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* after 24 hours.

	Control		*Amycolatopsis japonica*		*Sphingobium yanoikuaye*	
	Dead	Alive	Dead	Alive	Dead	Alive
Sytophillus spp. (pcs)	0 ± 1	10 ± 1	**4** ± 1	**6**± 1	**0** ± 1	**10** ± 1
Ryzopertha spp. (pcs)	0 ± 1	10± 1	**0** ± 1	**10** ± 1	**10** ± 1	**0**

4.7.4 Brine Shrimps Lethality assay using L-asparaginase produced from *Amycolatopsis japonica and Sphingobium yanoikuyae.*

The cytotoxicity of L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuye* was studied using brine shrimps lethality assay. Results obtained showed that L-asparaginase from *Amycolatopsis japonica* showed moderate toxicity of 47% on the shrimps at 5 μL concentration and 60% toxicity on the shrimps at 50uL concentration. High toxicity of about 80% of the enzyme was noticed on the shrimp in concentration of 500 μL. L-asparaginase from *Sphingobium yanoikuye* showed 43% toxicity on the shrimps at 5 μL concentration and 60% concentration at 50 μL concentration, 83.3% toxicity was noticed against the shrimp at 500 μL concentration (Table 4.19).

Table 4.19: Cytotoxic effect of L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* on brine shrimps.

	Amycolatopsis japonica	*Sphingobium yanoikuye*	**Control**
5uL	2 ± 1	5.66 ± 1	10± 1
50uL	4.33 ± 1	4 ± 0.1	10± 1
500uL	4.33 ± 1	1.66 ± 0.1	10± 1

4.7.5 Antileishmanial activity of L-asparaginase produced from *Amycolatopsis japonica and Sphingobium yanoikuyae.*

The effect of the crude L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* against Leishmania parasite was studied *in vitro* using *Leishmania donovani* and *Leishmania major*. It was observed that L-asparaginase from *Amycolatopsis japonica* showed the higher antileishmanial effect against *L. donovani* than *L. major*. The enzyme from *Amycolatopsis japonica* showed the highest inhibitory effect of 50% against *L. donovani* at concentration of 100nμg/mL and the lowest inhibitory (29%) was noticed against this parasite at 50 μg/mL concentration. 31% inhibitory effect against *L. donovani* at the 75 μg/mL concentration was also noticed i.e. increase in inhibitory effect as the concentration increases. L-asparaginase from *Amycolatopsis japonica* showed highest activity against *L. major* with growth inhibition of 22% at 100 μg/mL and no inhibitory effect of the enzyme was noticed when 25 μg/mL and 50 μg/mL was used. Inhibitory effect of 3% and 15% was noticed against the parasite at 50 μg/mL and 75 μg/mL concentration respectively. The study of antileishmanial effect of L-asparaginase from *Sphingobium yanoikuyae* revealed that the enzyme showed the inhibitory effect of 56% against *L. donovani* at 100 μg/mL concentration and showed 25% and 20% inhibitory effect at 75 μg/mL and 50 μg/mL concentration respectively. The enzyme showed 34% inhibitory effect against *L. major* at 100 μg/mL in all concentration (Table 4.20 and 4.21)

Table 4.20: Antileishmanial activity of L- asparaginase produced from *Amycolatopsis japonica*

	L. major			*L.donovani*		
	% Inhibition	% Inhibition	% Average	% Inhibition	% Inhibition	% Average
100µL	19.4444	23.6111	21.5	54.237	45.76271	50
75 µL	-9.72222	13.888	2.08	30.508	32.20339	31.393
50 µL	-51.3889	20.8333	-15.27	0	57.62712	28.856
25 µL	-34.7222	-47.2222	-40.92	-35.593	6.779661	-14.468

Table 4.21: Antileishmanial activity of L- asparaginase produced from *Sphingobium yanokuiye.*

	L.major			*L.donovani*		
	% inhibition	% Inhibition	%Average	% inhibition	% Inhibition	%Average
100µL	-4.16667	34.72222	15.27778	47.45763	64.40678	55.9322
75 µL	25	-26.3889	-0.69444	69.49153	-20.339	24.57627
50 µL	-8.33333	-12.5	-10.4167	40.67797	0	20.33898
25 µL	-20.83	-43.0556	-31.9444	-159.322	-59.322	-109.322

4.7.6 Antioxidant activity of L-asparaginase produced from *Amycolatopsis japonica and Sphingobium yanoikuyae.*

The antioxidant activity of L-asparaginase from *Amycolatopsis japonica and Sphingobium yanoikuyae* was performed by checking the scavenging activity of the enzyme through reduction of odd electron of nitrogen atom in (1,1, Diphenyl–2- Picrylhydrazyl (DPPH) to form hydrazine. The crude enzyme and the purified enzyme was used, from the result, it was observed that the crude L-asparaginase from *Amycolatopsis japonica* had 39% scavenging activity on the dye while the purified enzyme had 57.9%. The crude L-asparaginase from *Sphingobium yanoikuyae* had 12% scavenging activity on the dye while the purified enzyme had 16% (Figure 4.65).

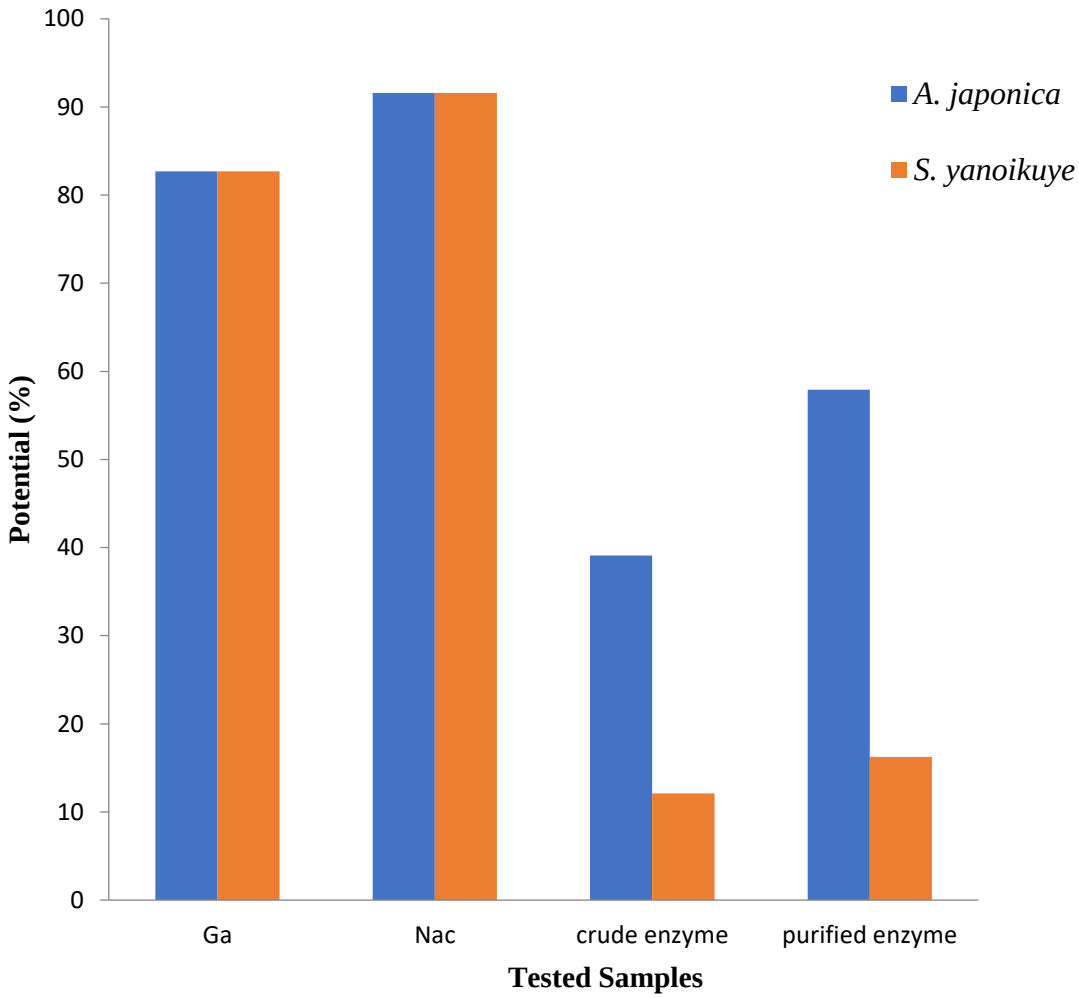


Figure 4.65: Antioxidant potential of L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae.*

4.7.7 *In vitro* anticancer activity of L-asparaginase produced from *Amycolatopsis japonica and Sphingobium yanoikuyae.*

Crude L- asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* was studied to elucidate their effectiveness on the growth inhibitory of three cell lines; Breast cancer cell line, 3T3 cell line (normal cell line) and colon cancer cell line using *in vitro* MTT assay (Figure 4.66 and table 4.22). The result showed that the crude L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* showed inhibitory effect on the growth of all the cell lines. It was observed that crude L-asparaginase from *Amycolatopsis japonica* showed more inhibitory activity against colon cancer cell line with growth inhibitory percentage of 56% and minimal growth inhibitory percentage of 7.6% against 3T3 cell line. 47.3% growth inhibitory percentage against breast cancer cell line was observed from crude L-asparaginase from *Amycolatopsis japonica*. Crude L-asparaginase from *Sphingobium yanoikuye* showed more inhibitory activity against colon cancer cell line with growth inhibitory percentage of 97% and low growth inhibitory percentage of 1.7% against 3T3 cell line. 46.8% growth inhibitory percentage against breast cancer cell line was observed from crude L-asparaginase from *Sphingobium yanoikuyae*.

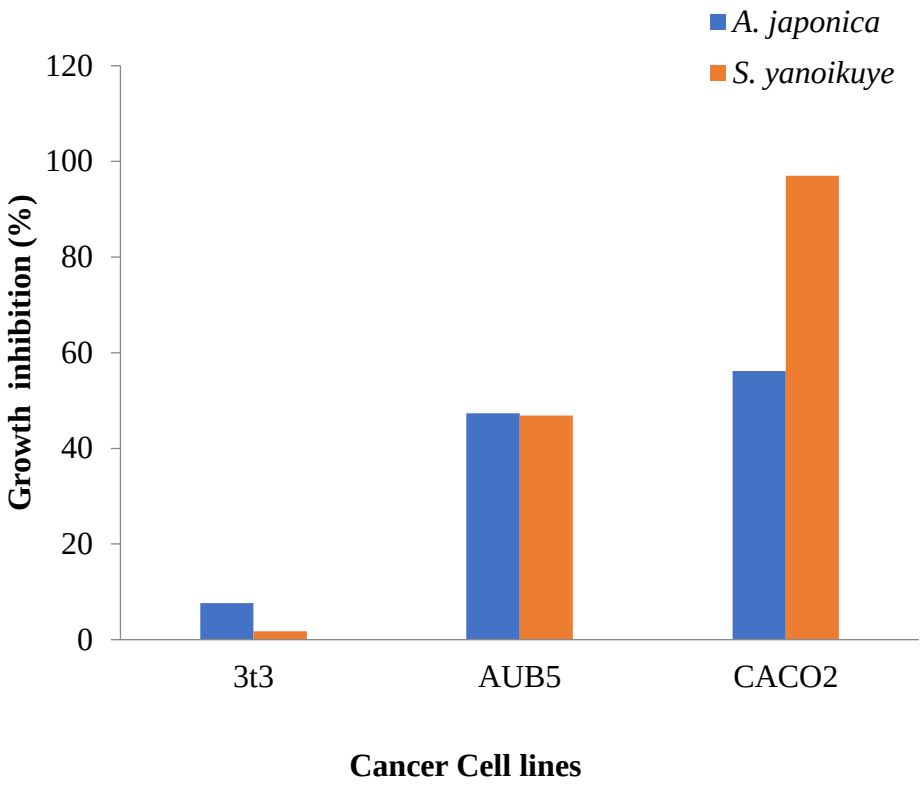


Figure 4.65: Anticancer potential of crude L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* on three cancer cell lines.

Keys:

3t3: Rat fibroblast cell line

AUB5: Breast Cancer cell line

CACO2: Colon cancer cell line

Table 4.22: Anticancer activity of L-asparaginase produced from *Amycolatopsis japonica and Sphingobium yanoikuyae.*

	3t3 fibroblast cell line				Breast cancer cell line				Colon cancer cell line			
	% Inhibition	% inhibition	% Inhibition	**Mean % Inhibition**	% Inhibition	% Inhibition	% Inhibition	**Mean % Inhibition**	% Inhibition	% Inhibition	% Inhibition	**mean % Inhibition**
A.japonica	10.9	5.700	5.70	**7.63**	44.58	43.66	53.74	**47.32**	90.33	78.11	-318.3	**56.14**
S.yanoikuyae	0.44	0.51	4.23	**1.73**	48.78	47.13	44.58	**46.83**	96.04	96.93	99.50	**97.49**

4.7.8 *In vitro* anticancer activity of purified L-asparaginase produced from *Amycolatopsis japonica and Sphingobium yanoikuyae.*

The purified L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* was tested on colon cancer cell line. The purified L-asparaginase from *Sphingobium yanoikuyae* showed more inhibitory effect on the colon cancer cell line with growth inhibitory percentage of 81% than purified L-asparaginase produced from *Amycolatopsis japonica* which gave growth inhibitory percentage of 46% against colon cancer cell line ($Caco_2$). The comparative analysis of anticancer potential of purified L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae.*on colon cancer cell line is shown in (Figure 4.66 and table 4.23).

Table 4.23: Anticancer activity of purified L-asparaginase produced from *Amycolatopsis japonica and Sphingobium yanoikuyae.*

	% Inhibition	% Inhibition	% Inhibition	Mean % Inhibition
A.japonica	45.05	48.119	0	46
S. yanoikuyae	94.752	91.881	56.53465	81.056

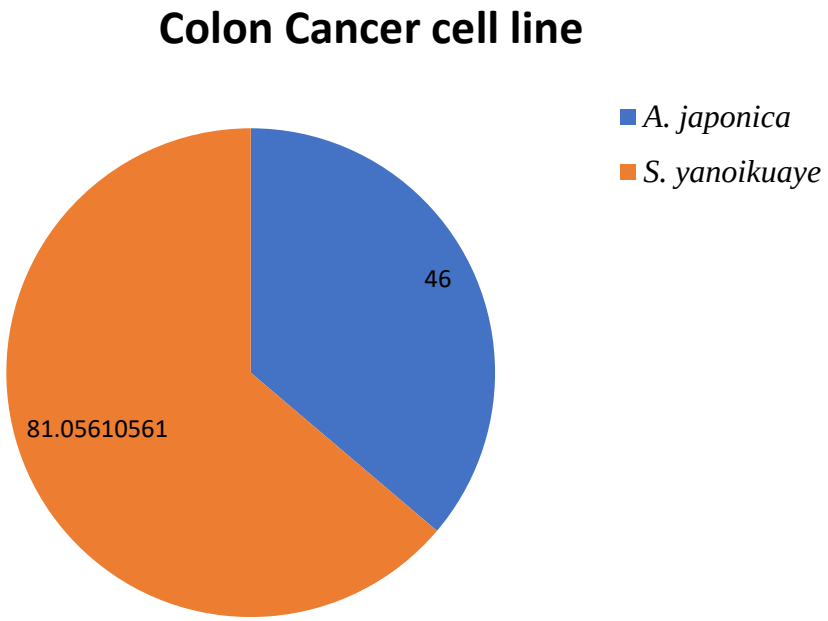


Figure 4.66: Comparative of anticancer potential of purified L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* on colon cancer cell line

4.7.9 *In vitro* anticancer activity of different concentration of purified L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae.*

Effect of different concentrations of purified L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* on colon cancer cell lines ($CaCo_2$) was studied. It was observed that, growth inhibition of 83.7%, 78.7% and 26.5% was noticed from L-asparaginase from *Amycolatopsis japonica* when the cell was treated with different concentration of 100 µL, 50 µL, and 25 µL respectively. The purified L-asparaginase from *Sphingobium yanoikuyae* gave growth inhibitory percentage of 98.6%, 76.7% and 48.8% against colon cancer cell at concentration of 100, 50 and 25 µL respectively. The amount of this enzyme that can kill 50% (IC_{50}) of the colon cells were calculated by using Ezi- fit software, and it was found to be 36 µL for L-asparaginase from *Amycolatopsis japonica* and 26 µL for L-asparaginase from *Sphingobium yanoikuyae*. Figure 4.67 and table 4.24 showed the effect of different concentration of purified L–asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* on colon cancer cell line. Plate 5 and 6 showed the pictoral and microscopic image of anticancer activity of L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* on colon cancer cell line.

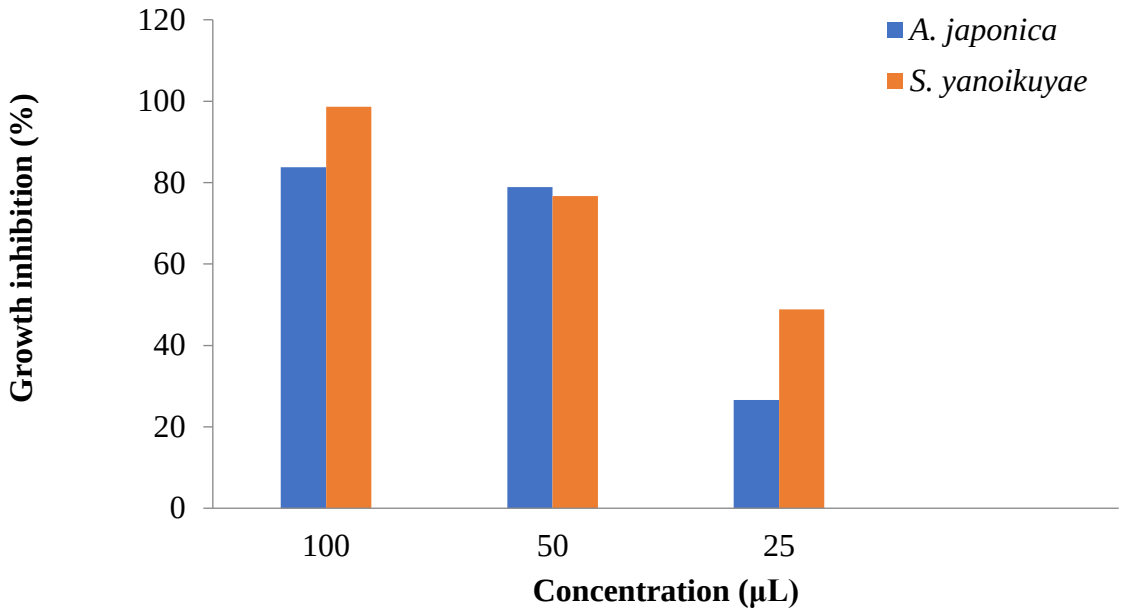


Figure 4.67: Anticancer potential of different concentration of purified L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* on colon cancer cell line ($CaCo_2$).

Table 4.25: Anticancer activity of different concentration of purified L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae*

	100µL			50 µL			25 µL			control		
	[illegible]	[illegible]	**Mean % Inhibition**	[illegible]	[illegible]	**Mean % Inhibition**	[illegible]	[illegible]	**Mean % Inhibition**	% Inhibition	[illegible]	**Mean % Inhibition**
A.japonica	[illegible]	[illegible]	83.74446553	[illegible]	[illegible]	78.8741303	[illegible]	[illegible]	26.5654649	77.04	[illegible]	74.6966
S. yanoikuyae	[illegible]	[illegible]	98.67172676	[illegible]	[illegible]	76.72359266	[illegible]	[illegible]	48.82985452	77.04	[illegible]	

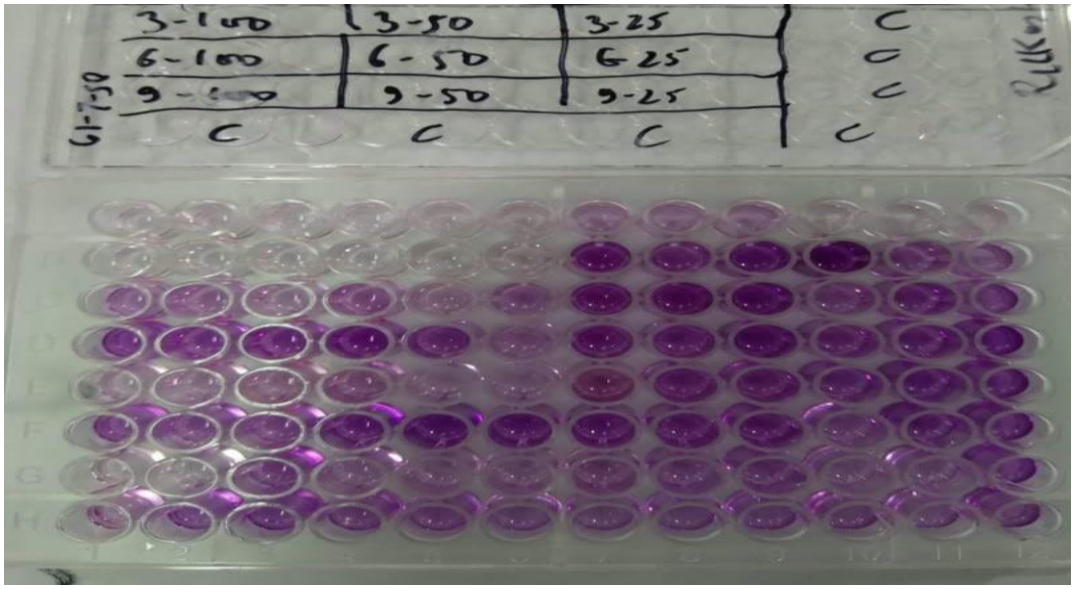


Plate 4.4; Image of anticancer activity of L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* on colon cancer cell line using MTT assay on 96 well bottom plate.

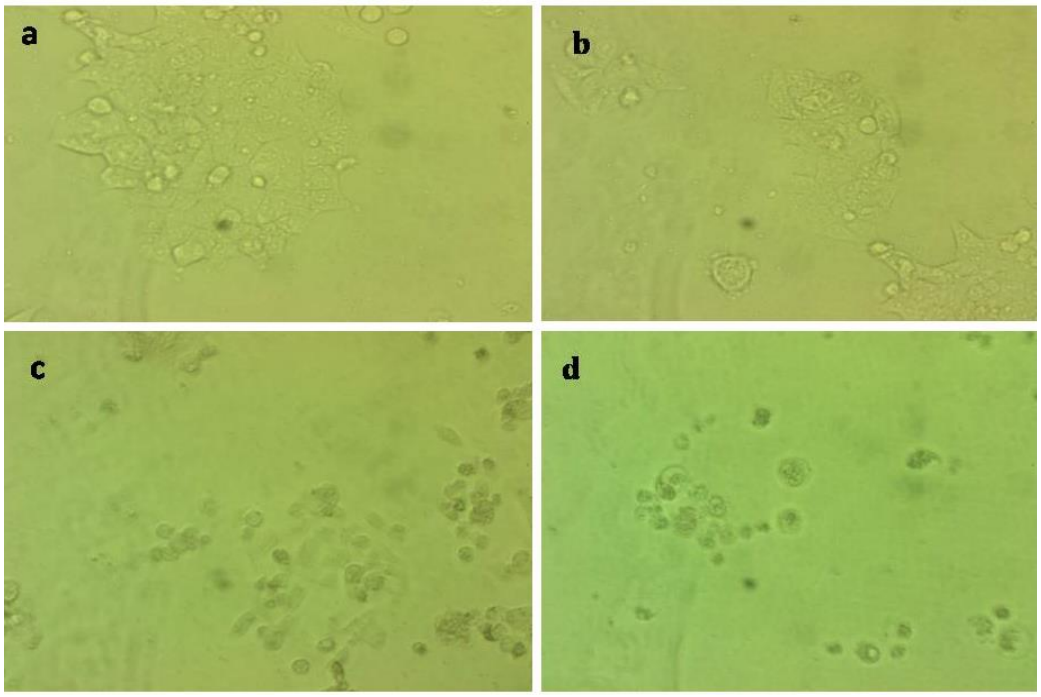


Plate 4.5; Pictoral image of anticancer activity of L-asparaginase from Amycolatopsis *japonica* on colon cancer cell line at different concentrations under fluorescent microscope.

Keys**:** **(**a) 100 µL; 83% cells were dead, **(**b) 50 µL; 78% cells were dead with few live ones, **(**c) 25 µL; Live cells with few dead cells up to 26%, (d) Control; Lives cells.

4.7.10 Acrylamide reduction potential of L-asparaginase produced from *Amycolatopsis japonica and Sphingobium yanoikuye.*

Fried mashed potatoes was treated with L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* and analyzed by gas chromatography mass spectroscopy (GCMS) to study their acrylamide reduction potential. Comparing the GCMS analysis of both treated and untreated samples, the acrylamide was present in both samples, but there was reduction of 11.89% in the formation of acrylamide in the mashed fried potatoes that was treated with crude L-asparaginase produced from *Amycolatopsis japonica* compared with untreated mashed fried potatoes. The mashed fried potato treated with crude L-asparaginase produced from *Sphingobium yanoikuyae* showed no formation of acrylamide after GCMS analysis compared with the untreated ones (Figure 4.69 and table 4.26).

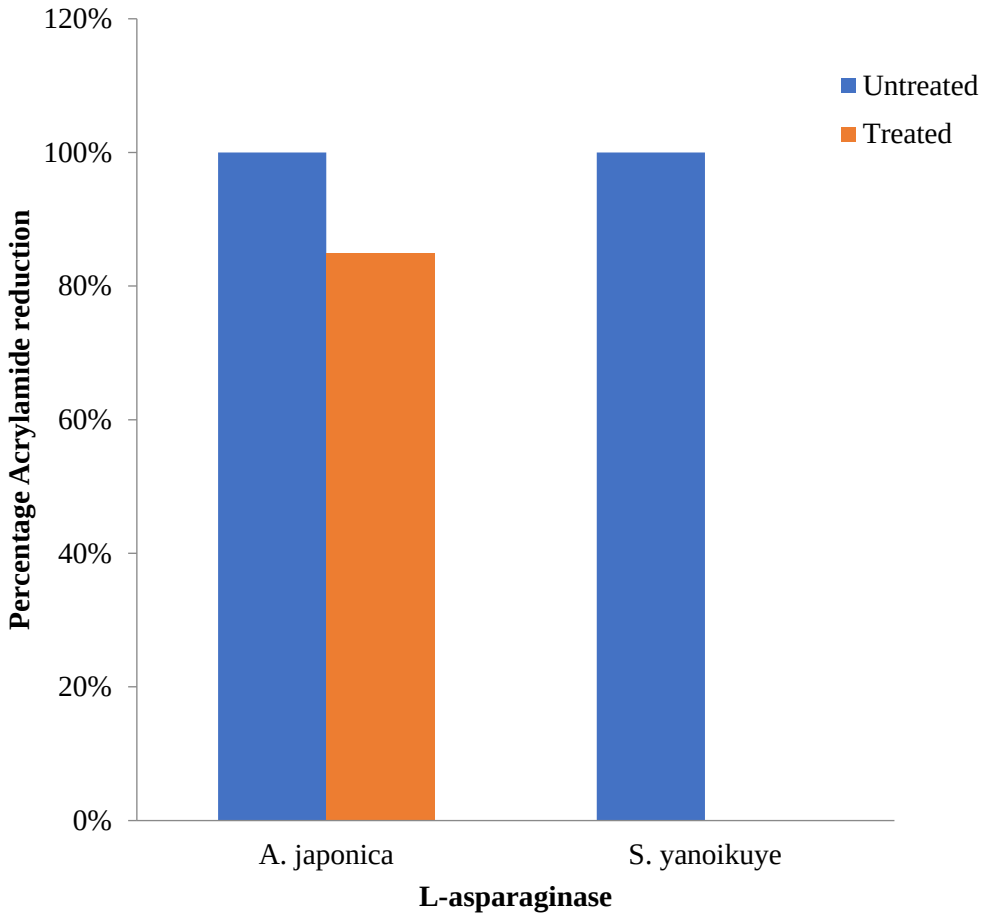


Figure 4.69: Acrylamide reduction potential of L-asparaginase produced from *Amycolatopsis japonica* and *Sphingobium yanoikuye* on food samples

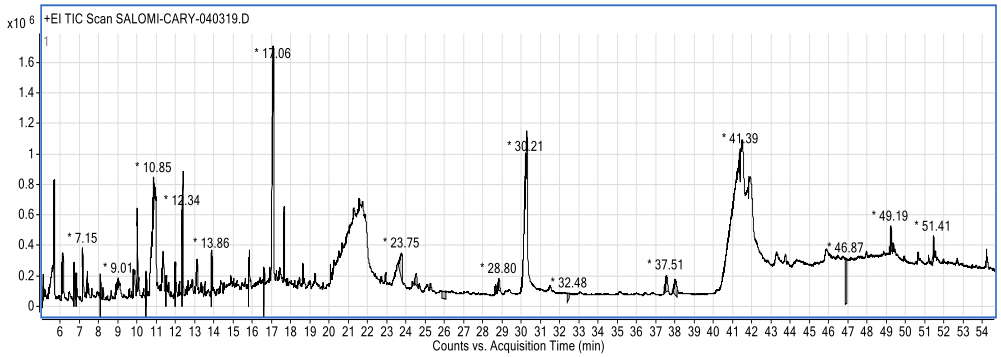


Figure 4.70: Gas chromatography mass spectroscopy (GCMS) spectra determining acrylamide content in fried untreated potato.

Table 4.26: peak analysis of the (GCMS) spectra determining acrylamide content in fried untreated potato.

Peak Number	RT	Area	Area %	Height	Width	Area Sum %	Height %
1	5.09	378057	25.91	177693	0.08	3.17	28.01
2	5.67	951695	65.23	605271	0.07	7.97	95.4
3	6.12	56862	3.9	89093	0.01	0.48	14.04
4	6.17	-1193	-0.08	0	0.01	-0.01	0
5	6.71	0	0	289383	0	0	45.61
6	6.81	179519	12.3	100885	0.01	1.5	15.9
7	6.83	0	0	221185	0	0	34.86
8	7.15	27472	1.88	128976	0	0.23	20.33
9	7.29	14029	0.96	16381	0.03	0.12	2.58
10	7.38	258051	17.69	150232	0.05	2.16	23.68
11	7.44	94484	6.48	64225	0.07	0.79	10.12
12	8.08	142315	9.75	53550	0	1.19	8.44
13	8.21	106435	7.29	60026	0.06	0.89	9.46
14	9.01	99239	6.8	41480	0.12	0.83	6.54
15	9.99	43924	3.01	117355	0.01	0.37	18.5
16	10.06	22996	1.58	0	0.02	0.19	0
17	10.46	0	0	227725	0	0	35.89
18	10.46	94689	6.49	442474	0	0.79	69.74
19	10.47	115082	7.89	84236	0	0.96	13.28
20	10.85	38904	2.67	137450	0	0.33	21.67
21	11.35	16081	1.1	92474	0	0.13	14.58

22	11.5	0	0	204730	0	0	32.27
23	11.62	120470	8.26	66937	0.06	1.01	10.55
24	11.98	27633	1.89	71993	0.01	0.23	11.35
25	11.98	0	0	291866	0	0	46
26	12.19	143795	9.86	81643	0.06	1.2	12.87
27	12.34	44948	3.08	233803	0	0.38	36.85
28	12.34	0	0	634427	0	0	100
29	13.1	8166	0.56	38158	0	0.07	6.01
30	13.51	70706	4.85	57145	0.05	0.59	9.01
31	13.86	0	0	354203	0	0	55.83
32	15.8	0	0	321814	0	0	50.73
33	16.59	0	0	259896	0	0	40.97
34	16.59	161490	11.07	468526	0	1.35	73.85
35	17.06	72897	5	355820	0	0.61	56.09
36	17.63	35106	2.41	90122	0	0.29	14.21
37	18.6	209767	14.38	119390	0.06	1.76	18.82
38	23.75	1089599	74.68	167362	0.25	9.12	26.38
39	24.49	513550	35.2	77205	0.25	4.3	12.17
40	25.24	211215	14.48	45239	0.16	1.77	7.13
41	25.91	683353	46.84	55260	0.21	5.72	8.71
42	28.8	433571	29.72	82351	0.18	3.63	12.98
43	30.21	496879	34.06	337454	0.03	4.16	53.19
44	32.48	317053	21.73	45395	0.1	2.65	7.16
45	37.51	835072	57.23	119143	0.19	6.99	18.78
46	37.98	1039823	71.27	102732	0.27	8.71	16.19
							99.99

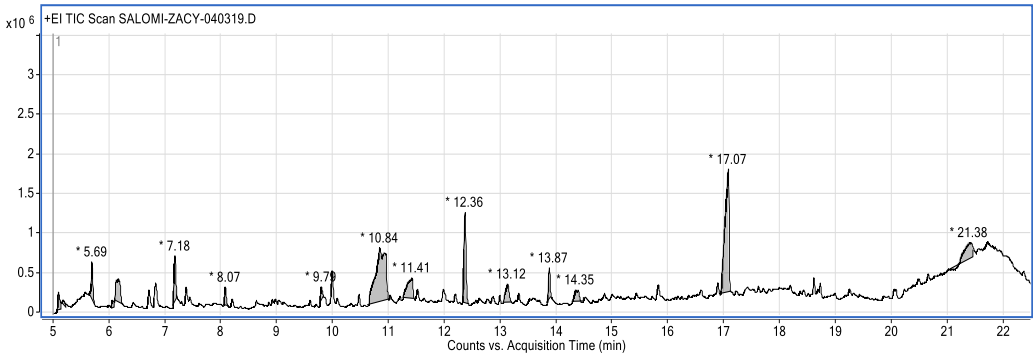


Figure 4.71: Gas chromatography mass spectroscopy (GCMS) spectra determining acrylamide content in fried potato treated with L-asparaginase produced from *Amycolatopsis japonica*.

Table 4.27: peak analysis of the (GCMS) spectra determining acrylamide content in fried potato treated with L-asparaginase produced from *Amycolatopsis japonica*.

Peak Number	RT	Area	Area %	Height	Width	Area Sum %	Height %
1	5.09	691214	0.49	239784	0.08	0.33	9.56
2	5.18	219422	0.16	64742	0.08	0.11	2.58
3	5.69	893587	0.63	467819	0.1	0.43	18.65
4	6.06	258179	0.18	106761	0.05	0.12	4.26
5	6.17	1598187	1.13	298262	0.16	0.77	11.89
6	7.18	1300712	0.92	588972	0.07	0.63	23.48
7	8.07	482909	0.34	234242	0.1	0.23	9.34
8	9.79	421299	0.3	189593	0.07	0.2	7.56
9	10.84	8531027	6.05	687082	0.37	4.11	27.39
10	11.41	1937661	1.37	267654	0.2	0.93	10.67
11	12.36	2613699	1.85	1149965	0.1	1.26	45.84
12	13.12	1093105	0.77	239120	0.16	0.53	9.53
13	13.87	859946	0.61	407526	0.08	0.41	16.24
14	14.35	899215	0.64	154573	0.21	0.43	6.16
15	17.07	7975235	5.65	1557812	0.2	3.84	62.1

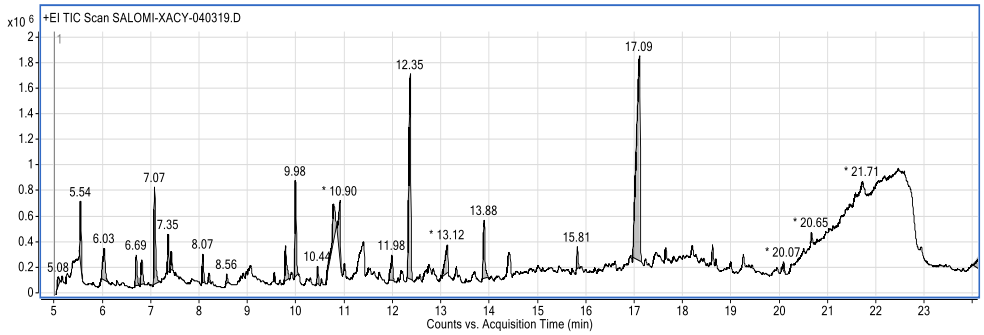


Figure 4.79; Gas chromatography mass spectroscopy (GCMS) spectra determining acrylamide content in fried potato treated with L-asparaginase produced from *Amycolatopsis japonica*.

Table 4.28: peak analysis of the (GCMS) spectra determining acrylamide content in fried potato treated with L-asparaginase produced from *Amycolatopsis japonica*.

Peak Number	RT	Area	Area %	Height	Width	Area Sum %	Height %
1	5.08	257241	2.79	80746	0.09	0.47	5.03
2	5.54	747536	8.1	473253	0.06	1.37	29.51
3	6.03	928170	10.05	253729	0.18	1.7	15.82
4	6.69	584088	6.33	247144	0.11	1.07	15.41
5	6.81	538381	5.83	198985	0.11	0.99	12.41
6	7.07	1927543	20.88	765197	0.16	3.53	47.71
7	7.35	511221	5.54	303274	0.06	0.94	18.91
8	7.42	228790	2.48	149699	0.05	0.42	9.33
9	8.07	472483	5.12	232970	0.08	0.87	14.53
10	8.2	130142	1.41	73858	0.06	0.24	4.6
11	8.56	167269	1.81	81890	0.08	0.31	5.11
12	9.55	106941	1.16	71654	0.05	0.2	4.47
13	9.78	788205	8.54	283603	0.11	1.45	17.68
14	9.98	1511717	16.37	758315	0.1	2.77	47.28
15	10.44	256729	2.78	144321	0.07	0.47	9
16	10.77	1700500	18.42	315827	0.22	3.12	19.69
17	10.9	861282	9.33	420506	0.1	1.58	26.22
18	11.98	640876	6.94	203140	0.12	1.18	12.67
19	12.35	4355151	47.17	1603893	0.14	7.99	100
20	13.12	960194	10.4	231079	0.12	1.76	14.41

21	13.88	1185417	12.84	462494	0.14	2.17	28.84
22	15.81	340681	3.69	183175	0.06	0.62	11.42
23	17.09	8524375	92.33	1601916	0.19	15.63	99.88
24	20.07	98951	1.07	62276	0.06	0.18	3.88
25	20.65	138307	1.5	88596	0.05	0.25	5.52
26	21.71	89464	0.97	47758	0.05	0.16	2.98
27	24.32	3444404	37.31	295604	0.41	6.32	18.43
28	25.07	752388	8.15	136911	0.19	1.38	8.54
29	25.09	248529	2.69	122294	0.06	0.46	7.62
30	30.24	8056589	87.27	904074	0.35	14.77	56.37
31	37.52	516835	5.6	81439	0.17	0.95	5.08
32	37.99	974550	10.56	98543	0.41	1.79	6.14
33	41.28	9232197	100	293701	0.94	16.93	18.31
34	41.73	1643519	17.8	116222	0.28	3.01	7.25
35	43.2	688919	7.46	93651	0.21	1.26	5.84
36	43.69	558195	6.05	92915	0.28	1.02	5.79
37	49.19	367252	3.98	120227	0.15	0.67	7.5

CHAPTER FIVE

DISCUSSION

5.1. Isolation, screening and identification

5.1.1. Total number of One hundred and forty-five isolates was collected from rhizosphere of 4 mature medicinal plants (*Azadirachta indica, Alstona boonei, Moringa oleace* and *Khaya senegalensis*), 21% from *Azadirachta indica*, 12% from *Alstona boonei*, 26% from *Khaya senegalensis* and 41% from *Moringa oleace*. The maximum number of bacteria was obtained in rhizospheric soil of *Moringa oleacea*. The number of bacteria from *Moringa oleacea* was higher than other soil samples which could be due to symbiont activity and availability of nutrient in the soil and on the species of plant. Similar observation was reported by Khamna and Yokota, (2009), who isolated different bacteria from Thai medicinal rhizospheric soil. Sharmal *et al.* (2021) suggest that, rhizosphere is a good reservoir of microorganisms perhaps due to high level of organic matter and types of the plant.

5.1.2. Screening for L-asparaginase producers

Sixty-seven isolates showed L-asparaginase producing ability by preliminary evaluation for L-asparaginase potential, six isolates showed L-glutaminase ability. The isolates were able to ultilise the L-asparagine and L-glutamine in the medium, thereby breaking it to L-aspartic acid and ammonia, the accumulation of ammonia in the medium led to increase in pH (Slightly acidic ph to alkaline pH), this was indicated by change in the initial colour of the medium (yellow) to pink due to an action of an indicator phenol red.

This submission is in line with the work of Nimkade and Barate, (2014), who screened isolates from soil sample for L-asparaginase activity. Also, Jayam and Kannam, (2014) reported *Streptomyces* species was screened for L-asparaginase producing ability. Saxena *et al.* (2015) reported that they screened four actinomycetes for extracellular glutaminase free L-asparaginase producing ability.

5.1.3. Identification of selected L-asparaginase producers

Six L-asparaginase producers with low glutaminase activity were selected and were molecularly identified using 16s rRNA sequence to be *Amycolatopsis japonica* (an actinomycetes), *Paenibacillus cineris, Sphingobium yanoikuyae, Stenotrophomonas pavani, Shingobacteium caeni* and *Actinomycetes bacterium*.

The isolation of these L-asparaginase-producing organisms is in agreement with the result of other researchers who isolated endophyte from soil rhizosphere. Souza *et al*. (2015), reported isolation *Themophila* spp. and *Amycolatopsis virids* from arid soil and *Amycolatopsis rhabdoformis* from tropical forest soil. *Amycolatopsis* sp. (L-asparaginase producers) has been reportd by Khamna *et al*. (2009) to be isolated from Thai medicinal plant rhizosphere. Also L-asparaginase producing *Stenotrophomonas matophila* was reported by Abdelrazek *et al*. (2020), Chowdhury, (2020) also reported *Stenotrophomonas Pavani* (a nitrogen fixing bacteria in soil). Wakil and Adelegan, (2015) reported isolation of L-asparaginase producing *Paenibacillus* from soil. Moorthy *et al.* (2010), isolated *Bacillus* sp. from the soil that produced extracellular L-asparaginase. Meyer *et al*. (2015) isolated *Shingobacterium rhizovivinum* from rhizospheric soil.

5.2. Studies of cultural parameters for L-asparaginase

5.2.1 Effect of pH on L-asparaginase production.

The initial pH play a major role on L-asparaginase production and the adaptability of different pH varies from one species of organism to another because, pH strongly affect the enzyme production and transport of various components across a cell membrane which will later help or enhance the formation of the product and growth of the producing organisms. This is evident from reports stating that acidity of the fermentation medium could inhibit the production of L-asparaginase (Narayana *et al.*, 2008, Lopez *et al.*, 2017).

In submerged fermentation experiment, the optimum growth and L-asparaginase production by *Amycolatopsis japonica* was at pH 7. However, the growth and L-asparaginase production by *Sphingobium yanoikuyae* was highest at pH 6. At pH 10, low yield of L-asparaginse was observed from *Amycolatopsis japonica* and there was L-asparaginase production from *Sphingobium yanoikuyae* at this pH. It was observed that growth and production of L-asparaginase by these organisms are

directly proportional, i.e. as the growth increases, so is the L-asparaginase production by these organisms.

The result obtained may be due to the fact that the pH of medium was suitable for the organism to grow, thereby facilitate the production of the enzyme. This is in agreement with the work of Narayan *et al.* (2008), Khamna *et al*. (2009), Kavitha and vijayalakshmi, (2012), they reported pH 7.0 as highest pH for the L-asparaginase production from *Streptomyces* sp F15, *Streptomyces plicatus* and *Streptomyces* AQB, respectively. Other actinomycetes species such as *Streptomyces aureofasciculus* LA2, *Streptomyces chattanoogenesis* LA-8, *Streptomyces* h*awaiiensis* LA15, *Streptomyces sp* LA-20, *S. canus* LA-29 and *S. olivoviridis* LA-35 showed maximum growth and L-asparaginase production at pH 7 to 8 (Hatanaka *et al*., 2011). Chandrasekkhar *et al*. (2012) recorded maximum L-asparaginase from *Aspergillus terrus* at pH 7.

5.2.2. Effect of temperature on L-asparaginase production.

One of the important enyironmental factors that influence production of L-asparaginase is temperature because it regulates the growth of microorganisms and the release of other secondary metabolites like enzyme.

Amycolatopsis japonica was able to grow well at temperature of 30^{o}C and the optimum L-asparaginase production was noticed between 30^{o}C and 35^{o}C. Production of L-asparaginase from *Sphingobium yanoikuyae* was high at 35°C. In both organisms, a gradual reduction of L-asparaginase production was noticed after 35^{o}C. This could be due to reduction in the growth of the organism and denature of the enzyme as a change in metabolic activities of the organism due to temperature. Sharma *et al*. (2014) and Lopez *et al.* (2017) recorded the optimum temperature for L-asparaginase production from *Streptomyces collinus* and *Streptomyces longsporus flavus* F-15 at 30°C.

Maximum temperature for the production of L-asparaginase from *Streptomyces plicatus* was reported by *Khamna et al.* (2009) to be 30°C. Sharma *et al.* (2014) reported maximum L-asparaginase production of *Streptomyces albidoflavus* at 35^{o}C. Similar observation was made in this study. Also, Khamna *et al.* (2009) observed the optimum growth and L-aspraginase production from *Amycolatopsis* sp. at 30 ^{o}C.

5.2.3. Effect of Incubation period on L-asparaginase.production

L-asparaginase.production by *Amycolatopsis japonica* began 3 days after the cell as grown. The highesr growth and L-asparaginase production by *Amycolatopsis japonica* was observed at after 7th day of incubation. The yield of the enzyme and the growth of the cells are directly proportional. Likewise the minimum L-asparaginase production from this organism was observed at day 14. Meanwhile, maximum growth and L-asparaginase production from *Sphingobium yanoikuyae* was noticed at day 14. From the above result, decrease in growth and L-asparaginase production by *Amycolatopsis japonica* as the days progresses could be due to accumulaton of toxic compound in the fermentation medium after a prolong incubation.

The maximum 7 days incubation period for L-asparaginase production is similar to that reported by Suttiham, (2009), He reported maximum production of L-asparaginase from *Amycolatopsis keratiphila* at day 7. Deshpande *et al.* (2014) reported that 6 days of incubation period favours the production of L- asparaginase from *Streptomyces ginsengisoli*. L-asparaginase production by *Fusarium solani* was noted on the 6th day by Srinival *et al.* (2006).

Narayan *et al.* (2008) reported that the growth and L-asparaginase production from *Streptomyces karnatakensis* and *Streptomyces albidoflavus* are directly proportional.

On the contrary, Khamna *et al.* (2009) and Kavitha and Vijayalashmi, (2010) reported maximum yield of L-asparaginase and biomass from *Amycolatopsis* CMU-H002 and *Streptomyces tendae* at day 3.

5.2.4. Effect of carbon sources on production of L-asparaginase.

Generally, in microbial fermentation processes, carbohydrates are mostly used as carbon. Microorganisms gained energy for their growth from light or through oxidation of the medium composition during industrial fermentation. Sources of carbon that is used in the formulations of media are to help to increase growth, which inturn leads to increased production of the enzyme. This is usually noticed during the synthesis of secondary metabolites.

Maltose came out to be the best carbon source that encourages maximum growth and enzyme production by *Amycolatopsis japonica*, followed by starch, and

sucrose in a submerged fermentation experiment. No yield of L-asparaginase production from *Amycolatopsis japonica* was observed when CMC was used as carbon source for production. However, starch and fructose supported L-asparaginase production by *Sphingobium yanoikuyae* and the CMC and groundnut shell as carbon sources for L-aspraginase production resulted in low yield of the enzyme.

The optimum production of L-asparaginase in the presence of maltose and starch from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* respectively, might be because maltose and starch enhances growth and biosynthesis of L-asparaginase by the organisms

The submission above is similar to the work of Narayan *et al.* (2008) and Desai and Hungund, (2018). They reported that optimum L-asparaginase production from *Streptomyces albidoflavus* and Actinomycetes isolate JRS-27 respectively was recorded when maltose was used as carbon source.

Contrary to this, maximum production of L-asparaginase from *Amycolatopsis keratiphila* was recorded by Khamna *et al.* (2009), when starch was used as carbon source. In addition, Venil *et al.* (2009) recommended sucrose as the most efficient carbon source for producing L-asparaginase by *S. marcescens*. They indicated that sucrose as an inexhaustible carbon source and its role in enzyme stabilization could explain its efficacy in L-asparaginase production. Darwesh *et al*. (2018) reported lactose as the best carbon source for L-asparaginase production by *P. carotovorum* and sucrose as the most efficient carbon sources for the productin of L-asparaginase from *Seratia marcesens*. Deshpande *et al.* (2014) reported maximum activity by *S. ginsegoli* when the medium was supplemented with glucose.

5.2.5. Effect of Nitrogen sources on L-asparaginase production.

Maximum growth of *Amycolaptopsis japonica* was noticed when yeast extract was used as nitrogen source and minimum growth was observed in the presence of ammomium sulphate. The highest L-asparaginase production was achieved with the medium that was supplemented with tryptone, followed by yeast extract and the minimum L-asparaginase production was found in ammonium sulphate. L-asparaginase synthesis from *Sphingobium yanoikuyae* was optimum when the

tryptone was used as nitrogen source and no yield of the enzyme in the presence of sodium nitrate and ammomium nitrate.

The obtained result is related to the result recorded by Khamna *et al.* (2009). They reported that yeast extract gave maximum L-asparaginase production when supplement in the medium for the production of L–asparaginase by *Amycolatopsis keratiniphila*. Also, Narayana *et al.* (2008) recorded that the best nitrogen source that that enhanced *Streptomyces albidoflavus* L-asparginase production was yeast extract. Kavitha and Vijalakshmi, (2012) reported that, the highest L-sparaginase production from *Nocardia levis* MK-VL113 was reported was in the production medium supplemented with yeast extract. Liu and Zajic, (1972) and El-Bessoumy and Ashraf, (2004) reported that high yields of L-asparaginase production by *Erwinia aroideae* and *E. carotovora* was in a medium supplemented with yeast extract, followed by tryptone.

Mc Tigue *et al.* (1994) reported that, the second affecting nutritional factor on microbial growth, performance and enzyme production by organisms is nitrogen. Qeshmi *et al.* (2018) reported that yeast extract is important for cell growth and biosynthesis of L- asparaginase. One of the nitrogen sources that is essential for growth of cells and syuthesis of L-asparaginase is yeast extract, nevertheless, high concentration of it limit the production of the enzyme (Qeshmi *et al.*, 2018).
On the contrary, Uzmal *et al*. (2016) reported sodium nitrate and ammonium nitrate has enhancer of L-asparaginase production from *Fusarium solani* and recorded lower enzyme production when the medium was supplemented with yeast extract. Deshpande *et al.* (2014) recorded peptone as the carbon source that favoured the maximum L-asparginase production by *Streptomyces ginsegoli*. Darwesh, *et al*, (2018) reported that, that influence of carbon and nitrogen source on L-asparaginase production is isolate-depended as they reported ammonium nitrate to be suitable source of nitrogen that gave high production of L-asparaginase from *P. carotovorum*

5.2.6. Effect of agitation on L-asparaginase production.

It was observed that shaking of production medium on a shaker at different revolution profoundly affected the production of L-asparaginase. Maximum growth of *Amycolatopsis japonica* and *Sphingobium yanoikuyae* was observed at 250 rpm and the lowest growth was noticed at 100 rpm. But compared to the enyzme production, the maximum yield was achieved at 150 rpm and the lowest yield at 100 rpm.

This is in synergy with report of Deshpande *et al.* (2014), they reported that low L-asparaginase production from *Streptomyces g*insengoli was observed at stationary state compared to the one kept at 120 rpm on the shaker.

The result gotten might be due quick depletion of the nutrient and disintegration of the enzyme due to rigorous agitation.

5.2.7. Effect of Innoculum size on L-asparaginase production.

The quantity of inoculums is another paramount factor that influences microbial metabolite production. Accelerated active growth and production of the metabolites can be achieved when appropriate innoculum size is used. This will reduce contamination from other microorganisms. The amount of innoculum will definitely affect the titre of the enzyme. It is imperative to optimize the volume of the innoculum so as to prevent too high or too low density of spores, which can result in inadequate growth of the cells, thereby resulted in inappropriate production of the product, or much amount of spores which lead to quick depletion of nutrient in the medium, thereby decreasing the quality of the end product.

A 1.5 $x10^8$ CFU/mL favoured the growth and L-asparaginase production by *Amycolatopsis japonica* and there was drastic decrease in the growth and the enzyme production with increase in numbers of cells used. On the other hand, 1.5 $x10^8$ CFU/mL encouraged most, the growth of *Sphingobium yanoikuyae* whereas, 6.0 $x10^8$ CFU/mL supported most, L-asparaginase production from the organism.

This may be due to moderate concentration of spores which allow the good growth of the organism and hence, enhance the production of the enzyme. The low yield observed in the higher concentration might be due to inability of the organism to grow which in turn leads to inability to produce enzyme.

5.2.8. Effect of subtrate concentration on L-asparaginase production.

Substrate concentration of 1% (w/v) was optimum for the growth of *Amycolatopsis japonica* while 1% (w/v) to 1.5% (w/v) substrate concentration was optimum for the production of L-asparaginase from this organism. Increase in yield with increase in substrate concentration was observed up till 1.5% (w/v). Substrate concentration of 0.5 - 1.5% favoured the growth of *Sphingobium yanoikuyae*, while 1.5% substrate concentration was optimum for L-asparaginase production from it. This might be because the medium was saturated, which makes its uncomfortable for the isolates to growth therefore limited the yield of the enzyme.

5.2.9. Effect of metal ions on L-asparaginase production.

Metal salts provide metal ions that are essential for cell mass formation and also act as cofactor for several biosynthetic enzymes (Yasser *et al.,* 2002). Production of L-asparaginase from *Amycolatopsis Japonica* and *Sphingobium yanoikuyae* in this study were best supported in the presence of magnesium ions.

This result obtained is in line with the observations of Kamble *et al.* (2012) that maximum production of L-asparaginase from *Pseudomonas aeruginosa* was in the presence of magnesium chloride.

5.3. Production, purification and characterisation of L-asparaginase produced by the selected strains.

5.3.1. Purification of L-asparaginase

The purification of enzyme is done to remove the cellular components and proteins present in the crude extract, which may change the activity and structure of enzyme to make the enzyme ready for analytical purpose (Fairooz *et al.,* 2021).

The produced L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* were partially purified. The activity of the enzyme and protein content were resolved into a single peak after the chromatographic profiling through gel filtration. This final purification step gave total enzyme activity of 1968.96 U, protein content of 26.69 mg with enzyme specific activity of 73.75 U/mg, 38.53 fold purification and recovery yield of 42.86% and total enzyme activity of 127.12 U, protein content of 18.70 mg with enzyme specific activity of 33.97 U/mg, 41.22 fold purification and recovery yield of 2.66% for *Amycolatopsis japonica* and *Sphingobium yanoikuyae* respectively.

Various researchers used almost similar method of purification for different enzyme, yet, the fold of purification and purification yields differ. This might be due to the fact that, various protein present in the fermentation medium have different interface (Meghavarnam *et al.,* 2015).

This result is in accordance to those reported by Sahu *et al.* (2007), who recorded purified enzyme with 18 fold and 1.9% recovery with specific activity of 13.57 U/mg from Actinomycetes strain L9. Kavitha and Vijayalaksh, (2010) recorded specific activity of 51.7 U/mg with a purity of 17.23 fold and the recovery was 30.5% from purified L-asparaginase from *Streptomyces tendae*. Narayan *et al.* (2008) recorded that L-asparaginase from *Streptomyces albidoflavus* showed purity of 99.3 fold with a final recovery of 20%. Lopez *et al.* (2017) reported purification of L-asparaginase of *Streptomyces* longsporusflavus (F-15) up to 30.5-fold with 19.1% recovery. Dhevagi and Poorani, (2006) reported that, L-asparaginase of *Streptomyces* sp. PDK2 showed a purity of 83 fold and 2.18% recovery. Also, Dias *et al.* (2016) reported 28.6 fold purification with yield of 6% recovery from A. oryzae. Kumar *et al.* (2011) recorded 42.02% yield 20.91 U/mg of specific activity from L-asparaginase produced from *P. carotovorum* by sephadex G-100 column chromatography.

Also, the specific activities of the enzymes increased with each successive purification step. Similar trend is also reported by Amena *et al.* (2009), Heinen *et al.* (2014) and Moharib, (2018).

5.3.2. Molecular weight of L–asparaginase

After purification by sephadex G-50, the fractions with highest activity were pooled together analysed by 12% Sodium deocyl sulphate polyacrylamide gel electrophoresis (SDS- page). In this work, the molecular weight of purified L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* were 37.5KDa and 65KDa respectively.

The molecular weight of L-asparaginase from different organisms differed as reported by various researchers. Dhevagi and Poorani, (2006) reported 140 kDa in *Streptomyces* sp. PDK2. 67KDa molecular weight of protein from *Streptomyces brollosae* was reported by El-Naggar *et al.* (2018). Narayana *et al*. (2008) reported 116 KDa in *Streptomyces.albidoflavus.* 80 kDa was reported in *Corynebacterium glutamicum* (El-Sabbagh *et al*., 2013), Bradoei. (2016) reported a single band of

protein from *Pseudomonas aeroginosa* to be around 35KDa. Dawesh *et al.* (2018) reported molecular weight of 34 and 40 kDa from *Pseudomona. carotovorum* and *Serratia. marcescens*, respectively. Kavitha and Vijayalaksh, (2010) reported a 97.4 kDa from *Streptomyces tendae.*

The differences in the molecular masses of L-asparaginase from different sources, even those from same microbial sources, is an indication of considerable diversity in the molecular weights of this enzyme.

5.3.3. Characterization of L-asparaginase

5.3.3.1. Effect of temperature on L-asparaginase acivity

Imperatively, temperature is one of the determinant factors that influence the activity of an enzyme. L-asparaginase (purified) from *Amycolatopsis japonica* showed good activity between 35°C to 55°C and behaved optimally at 45°C. It retained about 92% of it activity after 40 minutes of incubation. While that from *Sphingobium yanoikuyae* showed optimum activity at 45^{o}C and retained about 85% of its activity after 30 minutes of incubation.

This result is in agreement with the work of other scientists who recorded that optimum enzyme activity for L-asparaginase is between the temperatures of 35^{o}C to 45^{o}C. Borkotaky and Bezbaruah, (2002) recorded activity of *Pseudomonas stutzeri* MB-405 and *Erwinia* sp. L-asparginase to be maximal at 37°C and 35°C respectively. L-asparaginase from *Streptomyces gulbargensis* gave optimum activity at 40^{o}C (Amena *et al.,* 2010). Mesas *et al.* (1990) reported similar result for *Corynebacterium glutamicum* L-asparaginase. Dias *et al.* (2016) observed 50^{o}C temperature for maximum activity by *Aspergillus oryza.* Kumar *et al.* (2011), reported no significant loss of L-asparaginase activity purified from *Streptomyces radiopugnans* MS1, when the enzyme was pre-incubated at 40^{o} C for 60 min

The characteristics of this enzyme present it to be most useful for it to be removed completely from the body of a tumor patient that has been administered with it L-asparaginase *in-vivo*. The decrease in the enzyme activity with the increase in temperature maybe due to the denaturation of enzyme by destructing the three dimensional structure of protein and that cause a change in the active site which leads to inactivation of the enzyme at high temperatures.

5.3.3.2. Effect of pH on L-asparaginase activity

The maximum activity of the purified L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* was found in pH 8.0. The enzyme was more stable in the pH range 6.0 - 9.0 after 40 minutes of incubation at 37^{o}C, and nearly retained 50% of their activity at pH 8.0

Similar to this study, *Streptomyces* sp. PDK7 L-asparaginase gave it maximum activity at pH 8 - 8.5 (Dhevagi and Poorani, 2006). Basha *et al.* (2009) reported optimum activity of L-asparaginase from marine actinomycete at pH 7.0 and 8.0. Elshafei *et al.* (2012) reported that pH 8.0 was the optimal pH for L-asparaginase from *P. Brevicompactum*. L-apsaraginase extracted from *Pseudomonas stutzeri* MB L-asparaginase maximum activity was reported between pH 7.5 to 9.5 (Manna *et al.*, 1995).

At the opposite, the maximum activity of L-asparaginase from *Tetrahymena pyriformis* and *Corynebacterium glutamicum* was at pH 9.6 and 7.0 respectively (Triantafillons *et al.*, 1988, Mesas *et al.*, 1990).

L-asparaginase is an amidase enzyme and they have been reported to be stable and active at alkaline and neutral pH. Ramble *et al.*, (2005) reported that L-asparaginase of some bacteria is stable at the pH range of 5-9. Gaffar (2005) also observed that most L- asparaginase was stable at pH ranged (4.5-11.0) but more stability was observed when in alkaline pH. The enyme retained 50 % of its activity at pH 8.0 for 40 minutes. With this, the enzymes can be potential antitumor agent since stability of L-asparaginase at physiological pH has been reported to a determinant for antitumor activity.

5.3.3.3. Effect of substrate concentration on L-asparaginase activity

L-asparaginase of *Amycolatopsis japonica* had Vmax value of 0.13 mM and Km value of 0.43 U/mL and that from *Sphingobium yanoikuyae* had 0.37 mM L-asparagine and 0.32 U/mL.

Kotzia and Labrou, (2007) reported that *Erwinia chrysanthemi* L-asparginase had K_m value of (0.058 mM). K_m and V_{max} of purified L-asparaginase from *F. culmorum* ASP-87 were reported to be 3.1 mM and 0.77 μmol/ ml/min respectively. Km value of *P. brevicompactum* L-asparaginase was reported by Elshafei *et al.* (2012) to be 1.05 mM. Fernanda *et al.* (2016) reported that L-asparaginase from *A.*

oryzae CCT 3940 demonstrated high affinity for the substrate L-asparagine with Km and Vmax values estimated in 0.66 mol/L and 313 U/mL, respectively. Km value of 7.14 mM was recorded for L-asparaginase from *Erwinia carotovora* by Kramble *et al.* (2006). Higher Km values 6.6 and 7.0 mM for L-asparaginase from *Lupinus arboreus* and *Lupinus angustifolius*, respectively, has been reported (Chang and Franden, 1981). However, a slightly higher Km value of 12.5 mM were reported in *Aspergillus aculeatus* (Dange and Peshwe, 2011).

Summarily, from the above values, there exist great variations in the kinetic parameters of L-asparaginase from different sources. However, substrate concentration is another environmental factor that affects enzymes activity. The rate of an enzyme-catalysed reaction increases with increase in substrates concentration. Therefore the maximum velocity (Vmax) and Michaelis Constant (Km) of enzymes are usually determine in order to know the best substrates loading for enzymes meant to be applied. Vmax refers to the rate at which an enzyme converts its substrates to products in the presence of excess substrates, while Km refers to the concentration of substrates at which an enzyme acts at half its maximum velocity. Km is also a measure of the apparent substrates affinity of an enzyme.

From this study, the enzyme has high affinity to it natural substrate, which can be reasons for its degree of inhibition against cancer cell lines. Raha *et al.* (1990) noted that the effectiveness of an L- asparaginase enzyme against tumor is dependent on its affinity to its substrate.

5.3.3.4. Effect of metal ions on L-asparaginase activity

The influence of various metal ions on purified L-asparaginase activity was studied. Among the different metal ions tested, Mg^{2+} supported maximum activity of purified L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* while lowest activity of this enzyme was in the presence of Hg^{2+}. The activity of the enzyme from *Amycolatopsis japonica* was enhanced by 3%, and 70% of L-asparaginase activity from *Sphingobium yanoikuyae* was retained after 60 minutes of incubation in the presence of Mg^{2+}. Considerable loss of activity of L-asparaginase from both organisms was noticed in the presence of Hg^{2+}.

This report is in accordance with that of other researchers; Noura *et al.* (2018), reported that $HgCl_2$ was a potent inhibitor of *Streptomyces brollosae* NEAE-115 L–asparaginase. Of about 80%.activity of *Pectobacterium carotovorum* L-asparaginase was reported by Kumar *et al.* (2011) to be inhibited by Hg^{2+}. Moharib, (2009) reported that the activity of *V. Unguiculata* L-asparaginase was inhibited when Hg^{2+} and Zn^{2+} was present.$MgCl_2$, favours the activity of purified L-asparaginase from *Vigna radiate*, whereas, and mercuric chloride and zinc sulphate acted as inhibitors (Jayachandra, 2012). Badoei, (2015) reported Mg^{2+}, to be an enhancer of L-asparaginase from *Pseudomonas aeruginosa* strain SN004 and reported that in the presence of $HgCl_2$, there was total of enzyme activity from this organisms.

In the presence of Hg^{2+}, there was enzyme inhibition which might be due to the essential vicinal sulfhydryl groups (SH group) of the enzyme for productive catalysis.

Mg^{2+} ions increase the enzyme activity suggests that these metals ion can serve as co-factor, which can help to activate the enzymatic reaction. Mg^{2+} was thought to be the activating metal; Mg^{2+} may activate the substrate, bound directly to the enzyme-substrate complex. Mg^{2+} locks the enzyme-substrate complex in place and then rapidly causes release of the reaction products (Knape *et al.,* 2015*).* This corresponds to fast dissociation rates for the enzyme-product complex rendering more favorable substrate binding sites. Metal ions play a crucial role in maintaining the active configuration of the enzymes at elevated temperatures by protecting them against thermal denaturation (Kumar and Takagi, 1999)

5.3.3.5. Effect of inducers and inhibitor on L-asparaginase activity

The purified enzyme from *Amycolatopsis japonica* showed higher activity in the presence of Triton-X-100, tween 80 and EDTA. Whereas tween 80, and Triton-X-100 supported higher activity of purified L-asparaginase from *Sphingobium yanoikuyae.* Lower activity of the enzyme from both organisms was in the presence of ascorbic acid. L-asparaginase from *Amycolatopsis japonica* showed good stability within 60 minutes of incubation and retained up to 60% of its activity in the presence of EDTA. The activity of L-asparaginase from *Sphingobium yanoikuyae* was enhanced with 14%, 18% and 3% respectively after 20 minutes of incubation in the presence of TritonX-100, Tween 80 and EDTA,

This result obtained is in line with the work of Fernanda *et al.*, (2016) who reported slight decrease of around 30% of L–asparaginase from *Aspergillus oryzae* in the presence of EDTA. Noura *et al*. (2018) recorded that Tween 80 acted as activators for L-asparaginase activity from *Streptomyces brollosae* NEAE-115 and reported EDTA to be an inhibitor of L–asparaginase as slight decrease of about 37.55% in the activity of the enzyme was observed. Also, Moorthy *et al*. (2010) and Jayachandra, (2012) recorded inhibition of L-asparaginase activity from *Bacillus* sp. and *Vigna radiate* respectively by EDTA.

The fact that the activity of the enzyme was inhibited in the presence of some metal ion and was not inhibited in the presence of (EDTA) shows that the enzyme was not a metalloprotein. Direct and quick contact of enzyme with substrate sites seems to be increase by biosurfactant, and this might why Tween 80 supported the activity of the enzyme. (Castanon and Wilke, 1981*)*. Tween 80 enhanced substrate binding capacity and stability of enzymes under *in vitro* conditions (McAllister *et al.*, 2000).

5.3.3.6. Effect of Substrate specificity on activity of L-asparaginase

The L-asparaginase (purified) from *Amycolatopsis japonica* showed highest activity when L-asparagine was present with lowest activity in the presence of L-glutamine, and retained 60% of its activity in the presence of L-asparagine after 50 minutes of incubation. While that from *Sphingobium yanoikuyae* gave highest activity in the presence of L–phenylalanine followed L-aspartic acid and L-asparagine, and retained 68% of its activity in the presence of L-asparagine after 60 minutes of incubation.

The enzyme production is the complex chain reactions and is supported and induced by suitable substrates (Patro and Gupta, 2012). One of the properties of enzymes that make them useful as diagnostic tools is their specificity towards their substrate. The enzyme showed high specificity towards its natural substrate L-asparagine, very low specificity towards L-aspartic acid, while no activity towards L-glutamine.

This is in line with (Patro and Gupta, 2012) who reported that *Penicillium sp*. preferred L–asparagines as substrate and contrary to Dunlope and Roon (1975) who noted the increment in L-asparaginase production from *Penicillium sp* due to the addition of L glutamine or glutamate in the fermentation medium.

5.4. *In vitro* anticancer activity of L-asparaginase produced.

L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* showed more inhibitory activity against colon cancer cell line (Caco-2) and breast cancer cell line (AU5) but less on normal rat fibroblast (3T3) cell line. The enzyme exhibited more effectiveness on growth inhibition on colon cancer cell line but less on breast cancer cell lines.

The purified L-asparaginase from *Sphingobium yanoikuyae* showed more inhibitory effect on colon cancer cell line than that produced from *Amycolatopsis japonica*. The incubation of colon cancer cell line with gradual doses of L-asparaginase from these organisms led to a gradual inhibition in the cell growth with IC_{50} values of 36μL and 26μL from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* respectively.

Moharib, (2018), recorded that, L-asparginase from *Vigna unguiculata* seed have more anticancer effect against liver (HEPG2) and colon (HCT-116) but lower effective against cervical (HELA) and Breast (MCF7) cancer cell lines. studied the *in vitro* cytotoxicity of *Bacillus sp* R36 L-asparaginase against different cell lines and reported that, the enzyme have more cytotoxic effect on liver cancer cell line (Hep G2) than colon cancer cell line (HCT- 116) (Moharam *et al*., 2010). Fernanda *et al*., 2016 studied cytotoxic of L-asparaginase produced from purified L-asparaginase from *A. oryzae* CCT 3940 on broad range of human tumor cell lines (786-0 (kidney), NCI-H40 (lung, non-small cell), PC-3 (prostate),Type, U251 (glioma), UACC-62 (melanoma), HT29 (colon) and K562 (leukemia) at different concentration and reported that the enzyme completely inhibited the cell proliferation of these cell lines and did not inhibit the non-carcinogenic human cell line growth at the concentrations studied. Also, novel L-asparaginase from the 302 pathogenic strain *Helicobacter pylori* CCUG 17874 showed cytotoxicity against AGS and MKN-28 gastric 303 epithelial cell lines *in vitro* (Cappelletti *et al*. 2008). Aljewari *et al.,* 2010 studied L-asparaginase from *E.coli* isolated from urinary tract of infected patient against leukemia cancer cell line (U937 cancer cell line) and reported that the enzyme showed positive activity and high selectivity against U937 with IC_{50} of 0.5 ± 0.19 IU/mL.

The antineoplastic activity of the L-asparaginase produced by the isolated bacterial was performed based on the fact that lymphatic cells demand huge quantities of L-

asparagine in order to have rapid malignant growth as these tumor cells lack or have very low expression levels of L-asparagine synthetase and depend on the extracellular pool of this amino acid unlike normal cells. The presence of an external L-asparaginase enzyme in the growth medium causes depletion of asparagine due to the catalysis of the supplemented enzyme and kills tumor cells by depriving them of an essential factor required for protein synthesis (Prakasham *et al.*, 2009).

Tumor cells are destroyed by L-asparaginase without significant damage to normal cells. (Jalgaonwala and. Mahajan, 2014). This explains less inhibitory activity of this enzyme on 3T3 rat fibroblast cell because cancer cells are L-asparagine dependent, an amino acid essential for lymphoblasts growth. Non-cancerous cell has the ability to manufacture L-asparagine and cannot be affected by L-asparaginase treatment, because they contain L-asparagine synthase, cancerous cells do not have L-asparaginase synthase, and so they cannot produce L-asparaginase on their own, thereby affected when treated with L-asparaginase (Kotzia and Labrou, 2007).

5.5. Acrylamide reduction potential of L-asparaginase

There was reduction of about 11.89 % in the acrylamide content of the sample treated with L-asparaginase produced from *Amycolatopsis japonica* and no acrylamide was detected from the sample used on L-asparaginase produced from *Sphingobium yanoikuyae* when compared with the control (sample not treated with L-asparaginase).

The result obtained when L-asparaginase from *Sphingobium yanoikuyae* was applied might be because the enzyme has catalysed the L-asparagine in the potato before frying, thereby prevent the formation of acrylamide completely.

Pedreschi *et al*. (2007) studied the acrylamide content of fried potato and observed that the acrylamide formation was reduced with 60% when it was soaked inside the L-asparaginase solution. He reported that L-asparaginase reduced significantly the amount of asparagine, an important precursor of acrylamide formation.

CHAPTER SIX

6.1. SUMMARY

This study highlights the isolation of L- asparaginase producing bacteria from a rhizosphere of Medicinal plant. The organisms were identified as *Amycolatopsis japonica, Sphingobium yanoikuyae, Paenibacillus cineris, Sphingobacterium caeni, Actinomycetales bacterium* and*, Stenotrophomonas pavanii* by 16sRNA gene sequence. It was confirmed that rhizospheric soil is endowned with L- asparaginase producing bacteria with no or low glutaminase activity.

The nutritional and environmental factors have high impact on the growth and ability of the organisms to produce L-asparaginase. pH 7, 35^{o}C, 7 days, 150 rpm, M9 medium, 1.5% L-asparagine, maltose and yeast extract supported optimum L-asparaginase production from *Amycolatopsis japonica*. Whereas, pH 6, 35^{o}C, 14 days, 250 rpm, M9 medium, 1.5% L-asparagine, starch and tryptone supported optimum L-asparaginase production from *Sphingobium yanoikuyae.*

Partially purified L-asparaginase from *A. japonica* gave a total activity of 1968.98U with 26.696 mg total protein and a specific activity of 73.75 U/mg, 38.53 purification fold and recovery yield of 42.86% with a molecular weight of 37.5 KDa. whereas, Partially purified L-asparaginase from *Sphingobium yanoikuyae*.gave a total activity of 127.12 U with 18.70 mg total protein and a specific activity of 33.97 U/mg, 41.22 purification fold and recovery yield of 2.66% with a molecular weight of 65 KDa.

L- asparaginase from *A.japonica* showed good stability and activity when EDTA, triton and tween80 were present. Mg^{2+}, pH8, 45^{o}C and has the K_m value and V_{max} value of 0.13mM and 0.43U/mL respectively.. whereas L-asparaginase from *Sphingobium yanoikuyae* showed good activity and stability in the presence of tween 80 and triton X , Mg^{2+}, pH8, 45^{o}C and has the K_m value and V_{max} value of

0.37 mM and 0.32 U/mL. L- asparaginase from *A.japonica* showed high cytotoxicity against colon cancer cell line with IC_{50} value of 36 µL and 11% reduction of acrylamide formation in food, whereas, L- asparaginase from *Sphingobium yanoikuyae* showed high cytotoxicity against colon cancer cell line with IC_{50} value of 26 µL and 100% reduction of acrylamide formation in food .

6.2. **CONCLUSION**

From this result, it could be concluded that L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae* exhibited anticancer potential and reduced formation of acrylamide in fried starchy food.

6.3. RECOMMENDATION

It is recommended that L-asparaginase from these organisms could be used as drug to complement the ones currently in use, and further investigation on their vivo anticancer activity should be carried out.

6.4. CONTRIBUTIONS TO KNOWLEDGE

1. L-asparaginase-producing microorganisms with low glutaminase activity were isolated from plants rhizosphere
2. *Amycolatopsis japonica* and *Sphingobium yanoikuyae* L-asparaginase showed a high potential for attacking colon cancer cell *in vitro* and they could be a potential candidates for anticancer drug with less side effect.
3. To the best of my knowledge, this is the first report on L-asparaginase from *Amycolatopsis japonica* and *Sphingobium yanoikuyae.*

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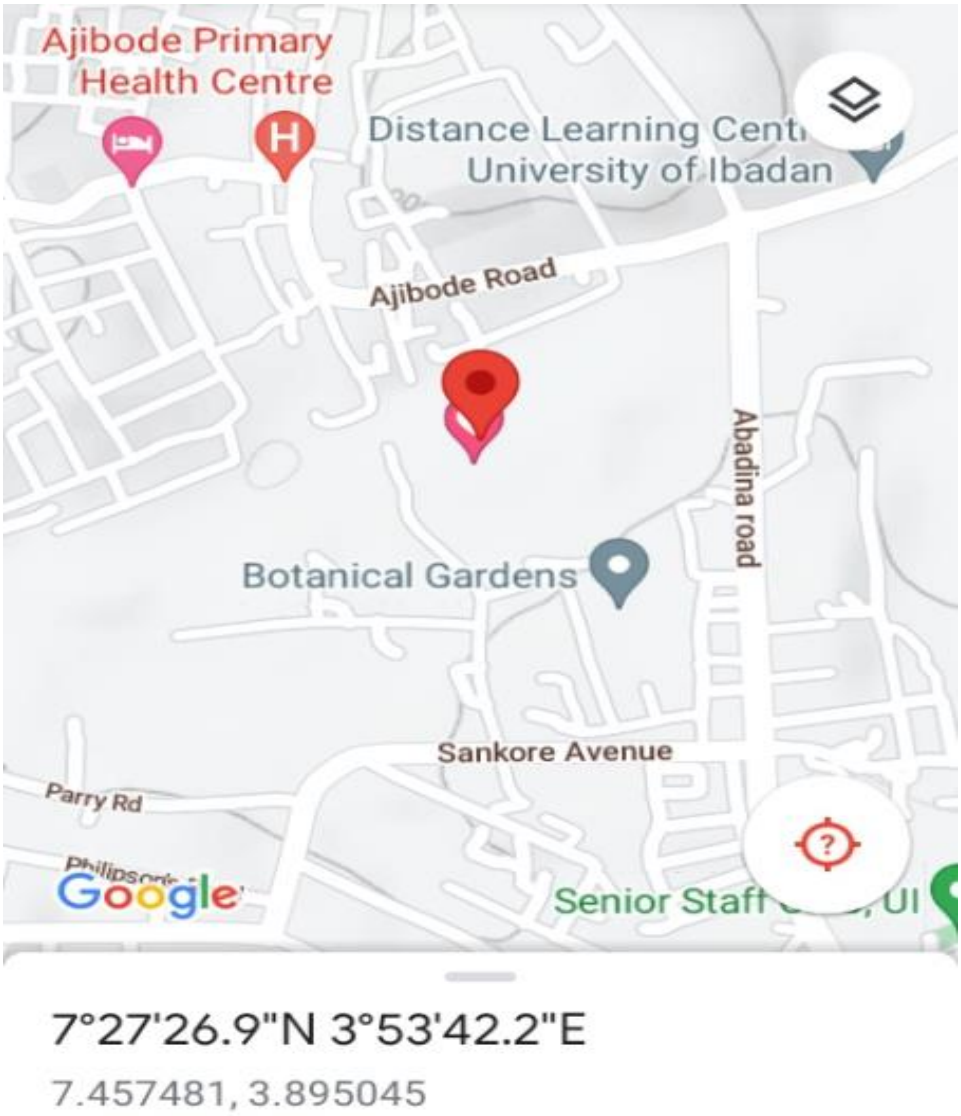
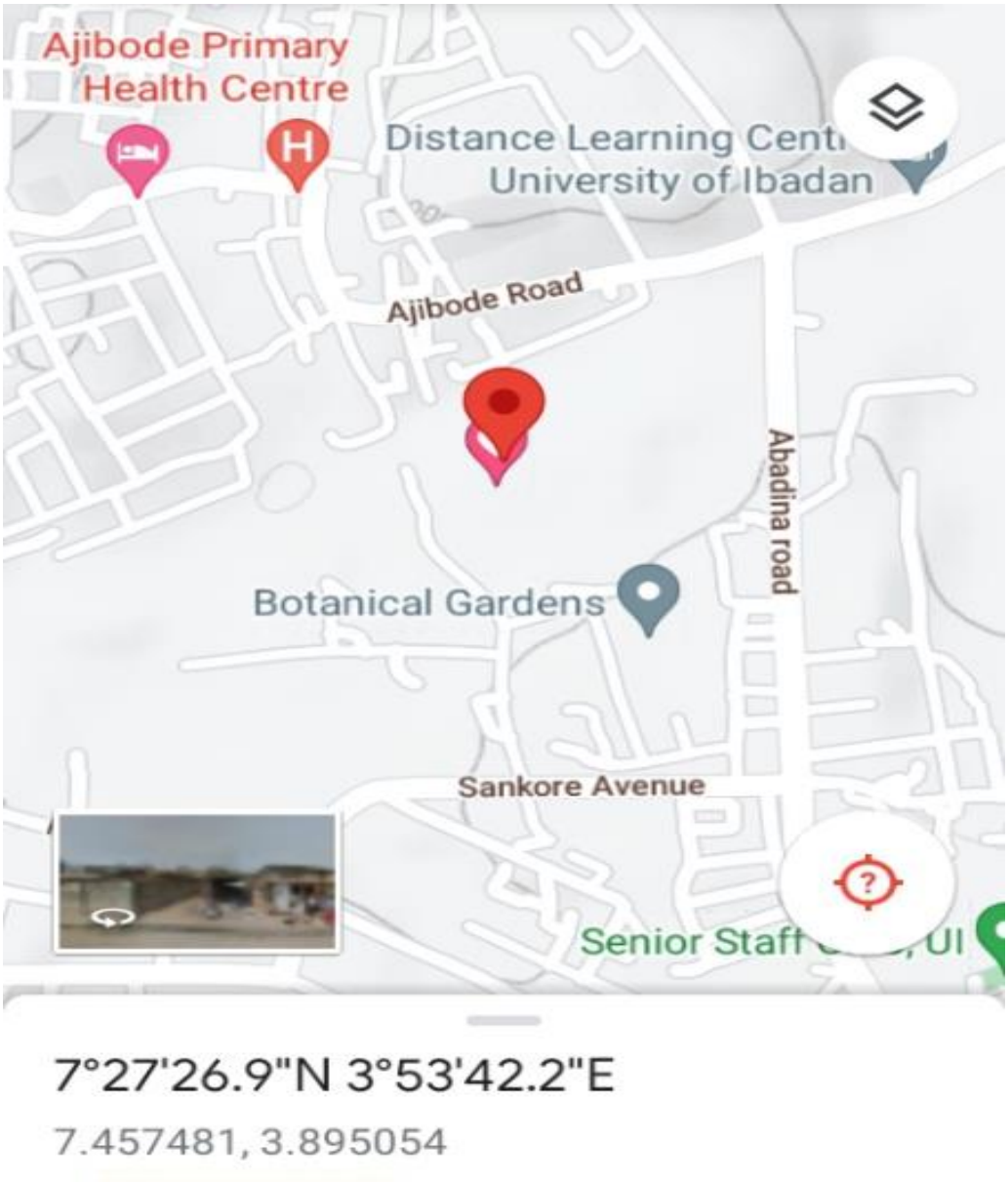
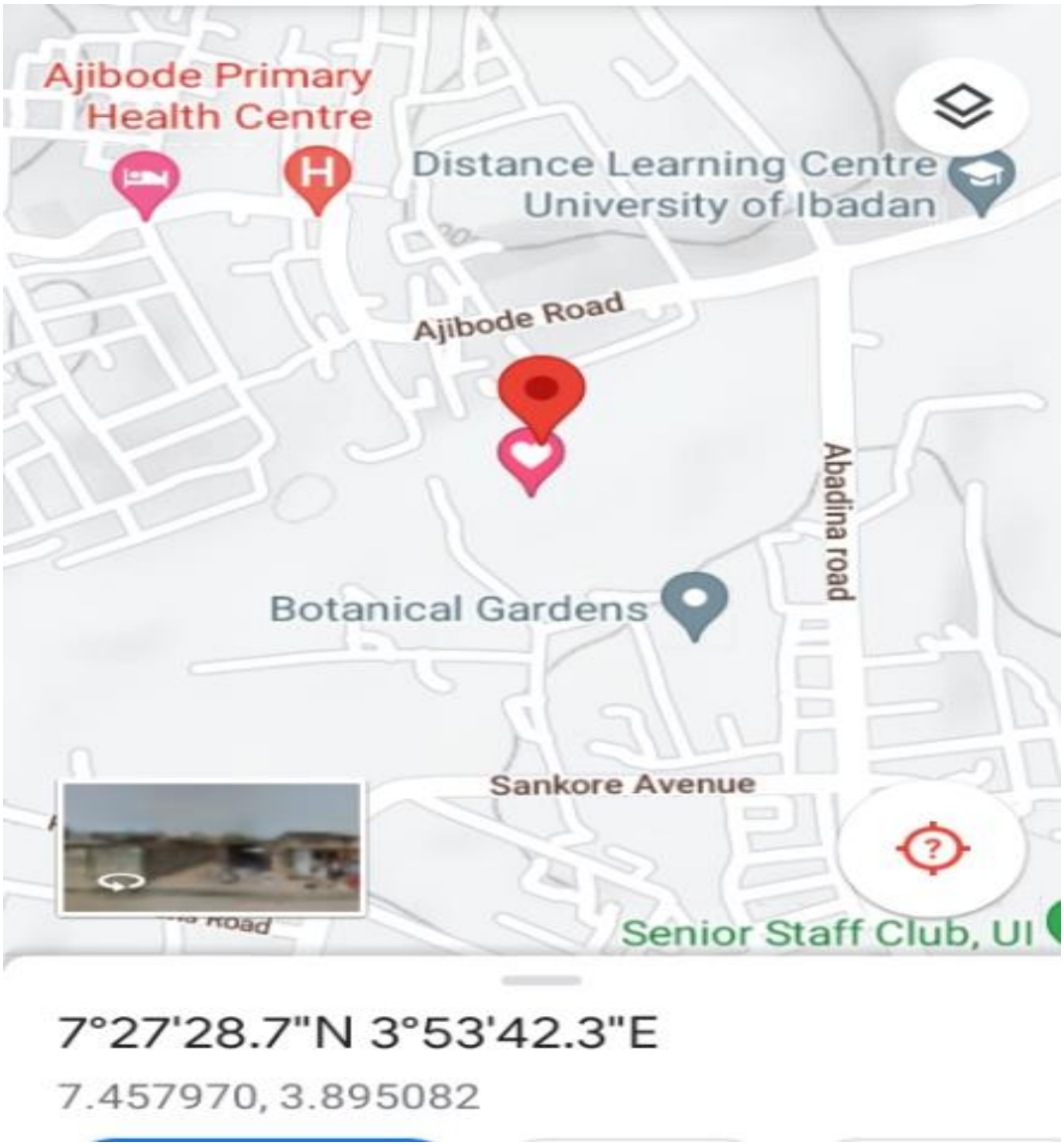
APPENDIX



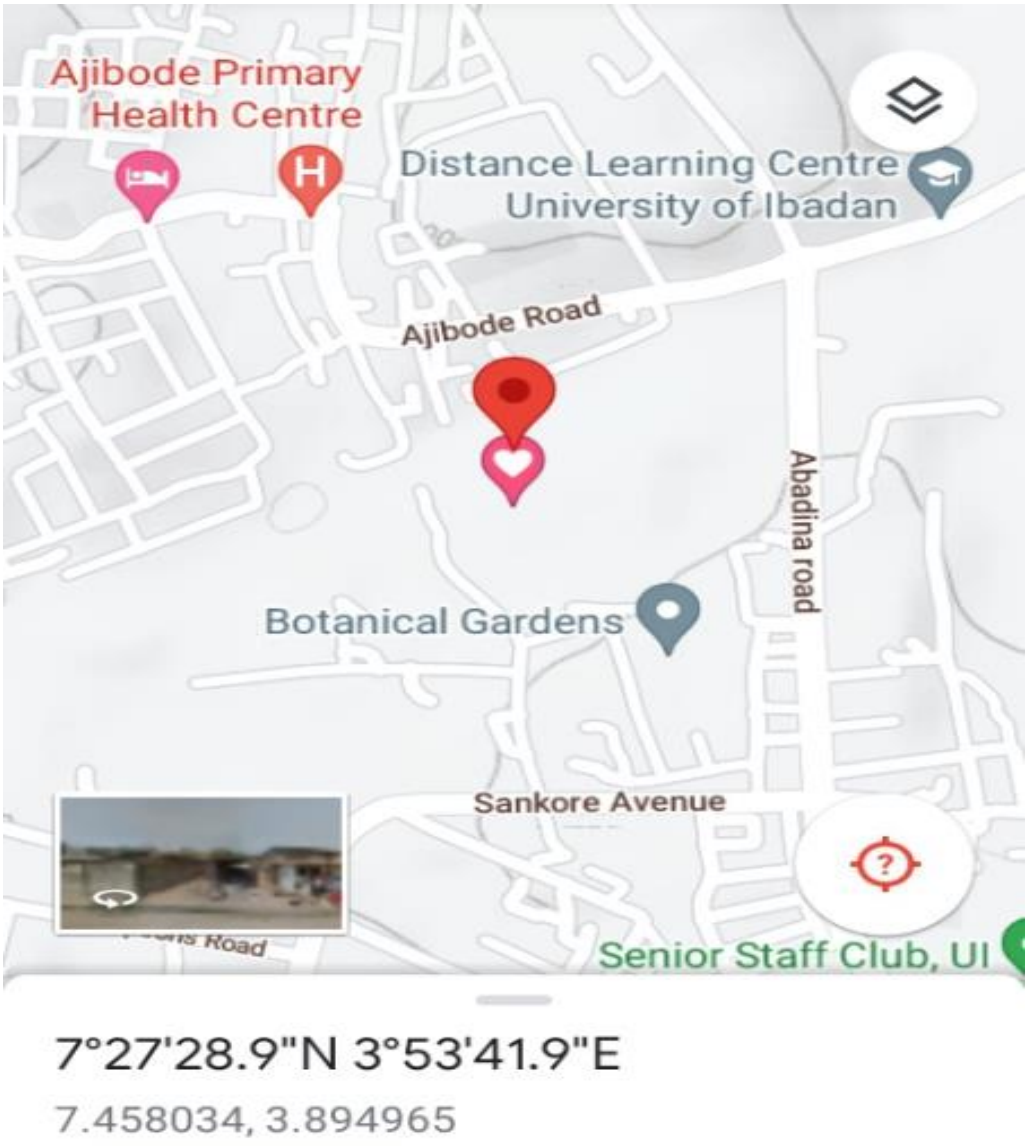
Appendix 1: The map shows the location of rhizospheric soil collection site (*Azadirachta indica*) within the Botanical Garden, University of Ibadan



Appendix 2: The map shows the location of rhizospheric soil collection site (*Alstona boonei*) within the Botanica Garden, University of Ibadan



Appendix 3: The map shows the location of rhizospheric soil collection site (*Moringa oleacea*) within the Botanical Garden, University of Ibadan



Appendix 4: The map shows the location of rhizospheric soil collection site (*Khaya senegalensis*) within the Botanical Garden, University of Ibadan