



Research Article

COMPARATIVE STUDIES ON EFFECT OF CARBON AND NITROGEN SOURCES ON L-ASPARAGINASE PRODUCTION

M. Mohamed Mahroop Raja^{1*}, A. Raja¹, S. Mohamed Salique², and P. Gajalakshmi³

¹Department of Microbiology, Jamal Mohamed College (Autonomous), Tiruchirappalli, Tamil Nadu, India.

²Department of Botany, Jamal Mohamed College (Autonomous), Tiruchirappalli, Tamil Nadu, India.

³Department of Microbiology, Dhanalakshmi Srinivasan College of Arts & Science for Women, Perambalur, Tamil Nadu, India.

*Corresponding author's email: mahroop_raja07@yahoo.co.in

Abstract

Marine actinomycetes sediment samples were collected from Gulf of Mannar coastal region, Kayalpatinam, located at Tuticorin district, Tamilnadu, India. Marine actinomycetes were isolated and evaluated for activity of L-asparaginase production. A total of 10 marine actinomycetes strains were isolated. Among 10 isolates, six were belongs to *Streptomyces* sp, three were belongs to *Micromonospora* sp and one was to *Micropolyspora* sp. Based on phenotypic characteristics, actinomycetes strains were screened for L-asparaginase production. *Streptomyces* sp KPMS5 and *Micromonospora* sp KPMS10 were showed large pink coloration on L-asparaginase production medium. The strains were further studied for maximum production and characterizations of culture condition of L-asparaginase enzyme were evaluated. Effect of substrate on L-asparaginase production was evaluated by enzyme assay. Maximum enzyme assay (1.4 mM) was observed on glucose followed by starch (1.12Mm) by *Micromonospora* sp KPMS10. In *Streptomyces* sp KPMS5 showed maximum of 1.25mM of enzyme assay on glucose substrate followed by lactose 1.17mM. Yeast extract was effectively used as substrate for maximum production of L-asparaginase by submerged fermentation. Further studies on purification and characterization are required to support the application of enzyme. The finding concludes isolates belongs to non-*Streptomyces* sp like *Micromonospora* sp is a potential novel source for L-asparaginase production.

Keywords: Leukemia; enzymes; L-asparagines; drugs; anticancer

Introduction

Many enzymes especially L-asparaginase has been attracted towards the use effective therapeutic agent against lymphocytic leukemia and many other types of cancer caused in human. Tumor cells is required to synthesis high amount of amino acid asparagines but it unable to synthesize their own, which is very closed for the better growth tumor cell, whereas non-tumor cell can synthesis their own asparagines and the cell growth is independent of its requirement (Pieters *et al.*, 2011; Jha *et al.*, 2012). Asparagine is a major component required for the synthesis of protein molecules. These can be synthesized by body itself, with in the cell by an enzyme called asparagines synthetase or can be taken from outside of the cell. L-asparaginase (L-asparagines amidohydrolase E.C.3.5.1.1) is an enzyme which converts L-aspartic acid and ammonia has been used as a chemotherapeutic agent in the treatment of acute lymphoblastic leukemia there by prevents tumor cells from rapid malignant growth for over 30 years. These enzymes are selectively killed by L-asparagine deprivation. The clinical action of this enzyme is attributed to the reduction of L-asparagine (Amena *et al.*, 2010). L-

asparaginase production by microorganisms are basically used in clinical practices because the diversity of microbial population is being explored for gathering novel sources of L-asparaginase production with less side effects and better treatment (Geckil and Gencer, 2004).

Screening of microbial sources for L-asparaginase activity has been greatly intensified and well documented in filamentous fungi, yeast and bacteria like *E.coli*, *Vibrio succinogenes*, *Erwinia carotovora* (Deokar *et al.*, 2010) and *Bacillus* sp. Among the fungi, *Mucor* sp., *Penicillium* sp., and yeast like *Candida utilis* have been proved to be potential producers of this enzyme (Joseph and Rajan, 2011).

Actinomycetes have gained special importance as the most potent source of bioactive secondary metabolites. Actinomycetes are group of thread like bacterial and look like fungi with high G+C content which form branching filaments or hyphae and asexual reproduction (Peela *et al.*, 2005). Some actinomycetes, *Streptomyces karnatakensis*, *Streptomyces griseus* (Dejong, 1972), *Streptomyces venezuelae*, *Streptomyces longsporusflavus*, *Streptomyces*

gulgargensis, marine *Streptomyces* sp PDK2 and *Nocardia* asteroids (Gunasekaran *et al.*, 1995) have been reported to be a potential L-asparaginase synthesizer. Various scientists have described the role of L-asparaginase in the treatment of cancer and cancer biology. A vast amount of investment has been made for the enzyme to explore the new effective ways. This research focuses on the better understanding of the L-asparaginase production by marine actinomycetes.

Methodology

The isolation and identification of actinomycetes from marine sediments sample was collected from Gulf of Mannar coastal region, Kayalpatinam, located at Tuticorin district, Tamil nadu, India. The marine sediments were collected and processed by serial dilution. One ml of diluted sample was permitted in to the starch casein agar medium supplemented with cyclohexamide 10µg/ml. Suspected actinomycetes were purified and identified by spore and biochemical properties.

L-Asparaginase Production by Plate Assay (Gulati et al., 1997).

Screening of marine actinomycetes for L-asparaginase production was performed on minimal medium incorporated with phenol red indicator by plate assay. This is the qualitative method used for L-asparaginase. The enzyme L-asparaginase was acted as substrate for L-asparaginase production. After incubation, the phenol red indicator turns pink zone around the colonies due to formation of ammonia at alkaline pH which indicates the production L-asparaginase.

Optimization of L-Asparaginase Production

The effect of carbon sources such as glucose, starch, lactose, mannitol and xylose on L-asparaginase production with peptone and yeast extract nitrogen sources was evaluated by submerged fermentation. The minimal media was prepared with 1% concentration of above mentioned carbon source. Two set up was prepared and one set up was supplemented with peptone (0.1%) and another one with yeast extract. The growth rate and enzyme assay were performed after 24 h.

Purification of L-Asparaginase

The purification of enzyme was carried out by using crude enzyme extract (Distasio *et al.*, 1982). The enzyme purification was performed by ammonium sulphate precipitation. Finely powdered ammonium sulfate was added to the crude extract. The L-asparaginase activity was associated with the fraction precipitated at 80% saturation. The precipitated was collected by centrifugation at 9,000 rpm for 15 min and dissolved in 1M Tris-HCl buffer and dialyzed against the same buffer.

Total Protein Estimation

Estimation of protein was determined by using lowery et al method. A stock solution of standard protein, BSA at a concentration of 1000 µg/ml was made. From these aliquots, 0.2-1 ml of working standard at concentration of

100 µg/ml was taken in a test tube. All the test tubes were made up to 1ml with distilled water. 1 ml of FC reagents was added to each test tube. After 30 min of incubation, the absorbance was measured at 660 nm by using UV-Vis spectrophotometer.

Enzyme Assay

The enzyme assay performed on M-9 broth medium for L-asparaginase enzyme production. The selected marine actinomycetes were sub cultured and inoculated in to M-9 broth medium. All the production flasks were kept at rotary incubator shaker for 72 h. After incubation, 10 ml of the cell suspension was taken and centrifuged. Quantitative detection was carried out by Nessler reagent method. 0.5 ml of cell suspension, 1 ml of 0.1M sodium borate buffer (pH 8.5) and 0.5ml of 0.04M L-asparaginase solution were mixed and incubated at 37°C for 10 min. The reaction was then stopped by the addition of 0.5 ml of 0.1N trichloroacetic acid. The precipitated protein was removed by centrifugation and the liberated ammonia was determined by direct nesslerization. Each sample was individually mixed with 1ml of 1N NaOH and 0.2 ml of 0.1M EDTA was added. After 2 min, 0.5 ml of nessler's reagent was added and mixed well. After 5 min, the addition of nessler's reagent to the sample, the yellow color was read on 117 UV-Vis spectrophotometer (Systronics) and the optical density of the sample was recorded at 450 nm.

Results and Discussion

Totally 10 marine actinomycetes were isolated and identified as *Micromonospora* sp, *Streptomyces* sp and *Micropolyspora* sp (Table 1). All the marine actinomycetes were differing morphologically based on the color of colony, types of mycelium, spore and pigmentation. Among the ten isolates, six isolates were belongs to *Streptomyces* sp (60%) that are predominantly present in marine environments followed by three isolates were belongs to *Micromonospora* sp (30%) and one isolate to *Micropolyspora* sp (10%). The dominance of *Streptomyces* sp is plentiful source in marine sediments and are reported by many workers (Lu *et al.*, 2009; Hassan *et al.*, 2011). Most of the strains produced spiral chains of spore are often spirally coiled and two of them were produced retractile spiral spore on substrate mycelium indicates the genera of *Streptomyces* sp. Mono spore was observed on actinomycetes colonies were belongs to the genera of *Micromonospora* sp was produced branched, non-motile and mono spore present only on substrate mycelium. The aerial mycelium was unstained by sudan black and the spore surface was smooth whereas the substrate mycelium was rough and stained by sudan black. The aerial mycelia were initially white and turned to ash, grey and dull white or chalky white on starch casein agar. The *Micropolyspora* sp KPMS 4 produced chain of spore with fragmented mycelium. All the isolated marine actinomycetes found in gram positive cell wall type. The group of actinomycetes

was currently up dated with spore forming gram positive bacteria that form extensive branching substrate and aerial mycelia (Waksman, 1961).

Species among the genera were differentiated based on the physiological properties (Table 2). Among the *Streptomyces* sp, three were found in catalase positive (KPMS2, KPMS5 & KPMS8). All *Streptomyces* sp were utilized starch and in oxidase except KPMS3. Similarly, *Streptomyces* sp of KPMS3 and KPMS6 were urease positive others were failed to obtain urease reaction. Among the *Micromonospora* sp, KPMS1 were positive all types of physiological reaction. Similarly, KPMS9 was positive only in urease reaction and KPMS10 was negatively found in starch utilization. *Micropolyspora* sp KPMS4 was negatively found in catalase, oxidase, urease and partially positive in starch. Isolation and characterization of promising strain of marine actinomycetes research is a

major area for many years in worldwide (Laidi *et al.*, 2006). In this purpose, identification of genera and species of actinomycetes, besides morphological and physiological properties were performed for the potent identification of actinomycetes isolates (Kuster, 1972; Abbas, 2006). Out of 10 isolates, 3 *Streptomyces* sp designated as KPMS 5, KPMS 6, KPMS 8 and one *Micromonospora* sp KPMS 10 were produced melanoid pigments. Actinomycetes are characterized by the production of various pigments on natural or synthetic media. The pigments may be dissolved in to the medium or it may be retained in the mycelium. Actinomycetes were known to be produced number of secondary metabolites and moreover these metabolites included many pigments. Production of pigments by marine actinomycetes has been utilized as an important cultural characteristic in describing the organisms (Miyaura and Tatsumi, 1960).

Table 1: Morphology of Isolated Marine Actinomycetes

S.N.	Strain code	Spore morphology	Color of aerial mycelium	Cell wall type	Isolated Genus
1	KPMS1	Septate hyphae with monospore	Greenish grey	Gram positive	<i>Micromonospora</i> sp
2	KPMS2	Spiral chain of spore	White	Gram positive	<i>Streptomyces</i> sp
3	KPMS3	Long chain of spore	Ash	Gram positive	<i>Streptomyces</i> sp
4	KPMS4	Chain of spore on fragmented hyphae	Ash	Gram positive	<i>Micropolyspora</i> sp
5	KPMS5	Spiral chain of spore	Dark greenish ash	Gram positive	<i>Streptomyces</i> sp
6	KPMS6	Moderate length of chain of spore	Grey	Gram positive	<i>Streptomyces</i> sp
7	KPMS7	Long chain of spore	Sandal white	Gram positive	<i>Streptomyces</i> sp
8	KPMS8	Rarely branched spiral spore	Light grey	Gram positive	<i>Streptomyces</i> sp
9	KPMS9	Septate hyphae with monospore	Dull white	Gram positive	<i>Micromonospora</i> sp
10	KPMS10	Septate hyphae with monospore	Whites ash	Gram positive	<i>Micromonospora</i> sp

Table 2: Physiological characteristics of Isolated Marine Actinomycetes

S.N.	Name of the Isolates	Catalase	Oxidase	Starch	Urease	Melanoid pigments
1	<i>Micromonospora</i> sp KPMS1	+	+	+	+	-
2	<i>Streptomyces</i> sp KPMS2	+	+	+	-	-
3	<i>Streptomyces</i> sp KPMS3	-	-	+	+	-
4	<i>Micropolyspora</i> sp KPMS4	-	-	±	-	-
5	<i>Streptomyces</i> sp KPMS5	+	+	+	-	+
6	<i>Streptomyces</i> sp KPMS6	-	+	+	+	+
7	<i>Streptomyces</i> sp KPMS7	-	+	+	-	-
8	<i>Streptomyces</i> sp KPMS8	+	+	+	-	+
9	<i>Micromonospora</i> sp KPMS9	-	-	-	+	-
10	<i>Micromonospora</i> sp KPMS10	+	+	-	+	+

+ : Positive; - : Negative; ± : Partial

All the ten actinomycetes were screened for L-asparaginase production at 37°C for 24-72h. Among 10 isolates, two isolated were showed L-asparaginase activity on plate assay method by pink colour zones formation around the actinomycetes growth which were belongs to genera of *Streptomyces* sp KPMS5 and *Micromonospora* sp KPMS10. Further, these isolates were subjected to secondary screening of enzyme activity. The production of L-asparaginase by various microorganisms using phenol red dye for screening techniques has been reported by other researcher (Sarquis *et al.*, 2004). Marine *Streptomyces* is serving as good source of L-asparaginase enzyme and converts L-asparagine to L- aspartic acid and ammonia. It has been used as a chemotherapeutic agent (Fisher and Wray, 2002). Among them, *Micromonospora* sp KPMS10 showed higher activity. To the best of our knowledge, this is the first report on the production of L-asparaginase. The results was presented as marine actinomycetes are a good source of L-asparaginase productions .Our reports was accordance with other worker (Savitri *et al.*, 2003).

The *Streptomyces* sp KPMS5 was able to use different carbon and nitrogen sources. Each carbon source and nitrogen source were incorporated in to basal medium at 1% and 0.1% concentration respectively. Among carbon sources, the maximum growth rate was found on glucose given an OD 1.58 with yeast extract by *Streptomyces* sp KPMS5 and least growth rate in xylose (OD 0.12). Among carbon sources supplemented with peptone, maximum growth of *Streptomyces* sp KPMS5 was 1.28 OD value and a minimum of 0.1 OD value respectively for glucose and xylose (Fig. 1).

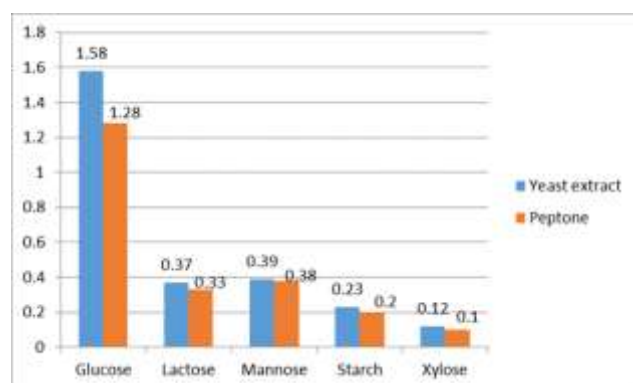


Fig. 1: Growth rate of *Streptomyces* sp KPMS5 on different carbon with yeast extract and peptone.

Similarly, *Micromonospora* sp KPMS10 to obtain the maximum growth rate was found on glucose at 1.18 OD value by and less rate in xylose at 0.11 OD value with yeast extract as nitrogen source (Fig. 2). In various carbon sources along with peptone, the maximum growth rate was observed in 1.02 OD value and a minimum of 0.1 OD value respectively for glucose and xylose. As previously described, yeast extract has been used as a potent nitrogen source for production of enzymes L-asparaginase by *Streptomyces albidoflavus* and *Streptomyces ginsengisoli*.

The significant growth rate of *Micromonospora* sp KPMS10 and *Streptomyces* sp KPMS5 were showed on glucose with yeast extract. Production of enzymes L-asparaginase was highly found in the presence of glucose as carbon substrate by a river isolate of *Streptomyces ginsengisoli* (Neelima Deshpande *et al.*, 2014). Among the carbon source, glucose, lactose and starch were found to be suitable for growth of actinomycetes whereas xylose was significantly affect the growth rate.

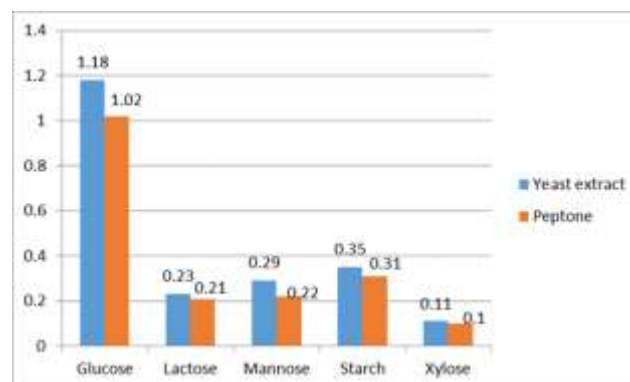


Fig. 2: Growth rate of *Micromonospora* sp KPMS10 on different carbon with yeast extract and peptone.

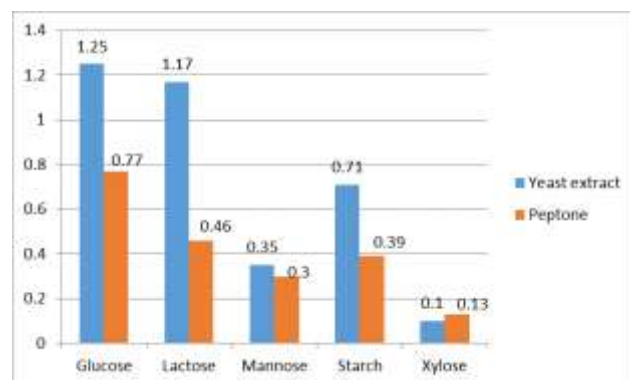


Fig. 3: L-asparaginase assay of *Streptomyces* sp KPMS5 with yeast extract and peptone

The enzyme assay of Nessler's method reveals that the strain *Micromonospora* sp KPMS10 was found to be maximum extracellular L asparaginase producer than *Streptomyces* sp KPMS5 under submerged conditions. The maximum enzymatic activity of *Micromonospora* sp KPMS10 was found in 1.4mM on glucose followed by 1.12mM on starch and minimum of 0.1mM on xylose with yeast extract. In peptone source, *Micromonospora* sp KPMS10 was found in 1.13mM on glucose followed by 0.96mM on starch and minimum of 0.1mM on xylose (Fig. 3). The *Streptomyces* sp KPMS5 was found a maximum of 1.25mM on glucose followed by 1.17 mM on lactose and minimum of 0.1 mM on xylose with yeast extract source. In peptone source, *Streptomyces* sp KPMS5 was found 0.77mM on glucose followed by 0.46 mM on lactose and least of 0.13 mM on xylose (Fig. 4). *Micromonospora* sp are potential actinomycetes to produce secondary metabolite with diverse chemical structure and biological activity. Currently, diazepinomicin is a unique farnesylated

dibenzodiazepinone and used as anticancer metabolite are derived from marine actinomycetes *Micromonospora* sp (Charan *et al.*, 2004). The potential actinomycetes exhibited both intra and extracellular production of L-asparaginase by *Streptomyces longsporusflavus* (Abdel-Fattah and Olama, 1998).

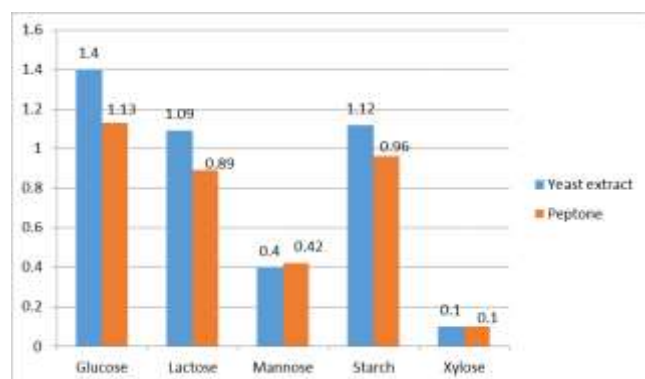


Fig. 4: L-asparaginase assay of *Micromonospora* sp KPMS10 with yeast extract and peptone.

Difference on production of L-asparaginase with yeast extract and peptone was observed and evaluated at 24h. Among the two nitrogen sources, maximum enzymatic activity was found on yeast extract source given by *Micromonospora* sp KPMS10 and *Streptomyces* sp KPMS5. Yeast extract has been reported as a good nitrogen source for the production of L-asparaginase by *Streptomyces albidoflavus* (Narayana *et al.*, 2008). L-asparaginase activity in *Streptomyces* sp isolated from the foregut and hindgut region of clam, *V. cyprinoids* (Mathew *et al.*, 1994; Koshy *et al.*, 1997). The maximum rate of L-asparaginase production by *Amycolatopsis* CMU-H002 was amended with starch as the carbon source (Khamna *et al.*, 2009). Among the carbon source, glucose and starch showed the immediate conversion of L-asparagines into ammonia was observed within 24 h under submerged condition. The higher enzymatic activity occurred in submerged fermentation by *Streptomyces* sp (Sahu *et al.*, 2007). Carbon sources except xylose and other sugar molecules showed L-asparaginase enzymes activity to be found after 48 h of incubation. The least L-asparaginase activity was found on xylose as from 0.1 mM by both tested actinomycetes.

The L-asparaginase was partially purified by ammonium sulphate precipitation and analyzed further for characterization of protein. The total protein content was found to be 2 mg/ml and 1.8 mg/ml for *Streptomyces* sp KPMS5 and *Micromonospora* sp KPMS10. The production and purification of L-asparaginase by marine actinomycetes *Streptomyces tendae* TK-VL-333 isolated from sediment samples of Parangipettal and Cochin coastal region in South India has been reported (Kavitha and Vijayalakshmi, 2010). The isolate PDK7 and PDK8 from Parangipettal and Cochin coastal region in South India, were gave potential L-asparaginase activity (Dhavalala *et al.*, 2006). Similarly,

among 10 actinobacterial strains, three isolate S3, S4 and S8 belongs to *Streptomyces* sp were showed extracellular production of anti-leukemic enzyme L-asparaginase (Basha *et al.*, 2009). Several terrestrial *Streptomyces* are capable of producing detectable amounts of L-asparaginase. There are limited reports on production of L-asparaginase from marine *Streptomyces* like *Streptomyces aurantiacus* (Gupta *et al.*, 2007). *Streptomyces* and *Micromonospora* sp are distributed in both marine and terrestrial environments. They have unique features to produce novel bioactive metabolites such as antibiotics, enzymes and antitumor compounds (Mitchell *et al.*, 2004).

Conclusion

Marine actinomycetes are one of the most eminent secondary metabolites synthesizers and are very important in industrial point of view. Enzyme after antibiotics are most important metabolites derived from actinomycetes. This study concludes that *Streptomyces* sp and *Micromonospora* sp is an efficient producer of L-asparaginase. Marine actinomycetes are well organized synthesizer of both extracellular and intracellular L-asparaginase and holds potential for treatment of acute lymphocytic leukemia and cancer in other types. With the increasing advancement in modern science, there would be a greater demand for new metabolites synthesized from marine action bacteria in future.

Acknowledgement

We thank the Department of Microbiology, Jamal Mohamed College (Autonomous), Tiruchirappalli-620020, for supporting and fulfilling all the needs to carry out this work.

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