Isolation And Identification Of L-Asparaginase, An Anticancer Drug Producing Bacteria Occurring In Soil

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Abstract: Asparaginase (ASNase) is a commercially important enzyme which is used in pharmaceutical industry as anticancer drug to cure leukemia and lymphoma. It is also used in food industries especially during the preparation of baked foods to prevent the formation of a carcinogenic compound acrylamide. Many microbes are exploited to produce ASNase. In the present work soil samples from slaughter house, fish market and garden were collected and processed in the laboratory to isolate ASNase producing bacteria. Eight ASNase producing bacteria were isolated and identified till genus level based on microscopic examination and biochemical characterization. Two bacteria positive for ASNase were isolated from slaughterhouse soil sample and identified as Serratia (A1) and Proteus (A2) species. Four bacteria positive for ASNase production isolated from fish market soil were identified as Pseudomonas (B1), Staphylococcus (B2), Bacillus (B3) and Escherichia (B4) species. From garden soil two bacteria were found to be positive for ASNase production and they were identified as Bacillus (C1) and Acinetobacter (C2). Based on pH change due to ASNase production in the medium Bacillus (B3) isolated from fish market soil was identified as efficient producer of ASNase when compared to the other bacterial isolates.

Keywords: Asparaginase, anticancer, leukemia, lymphoma, food industries, acrylamide, ASNase producing bacteria.

1. INTRODUCTION

The L- asparaginase (ASNase) enzyme produced by microbes gained medical importance as it can be used as a drug for the treatment of certain types of human cancers specifically acute lymphoblastic leukemia and Hodgkin's lymphoma [1,2]. The ASNase is advantageous over other anticancer agents as it is easily degradable, non-toxic and it can be easily routed to the target site. Other anticancer agents when used for treatment cause pain to patients and moreover they are expensive [3]. L-asparagine is one of the non-essential amino acids required for protein synthesis in living cells. In cancer patients both normal and tumour cells need to synthesize L-asparagine. The enzyme, asparagine synthetase catalyzes the synthesis of L-asparagine. The normal cells contain optimum levels of asparagines synthetase whereas tumour cells are devoid of the same enzyme. Hence, tumour cells depend upon blood serum for L-asparagine. When ASNase is administered into the patients it hydrolyzes L-asparagine present in serum into aspartic acid and ammonia. This leads to the depletion of L-asparagine in serum. As a result, L-asparagine is not available to tumour cells and eventually they die [4, 5, 6]. A wide variety of microorganisms have been reported to produce ASNase which include E.Coli, Serratiamarcescens, Pseudomonas aeruginosa, Bacillus circulans, Streptomyces albidoflavus, Streptomyces gulbargensis,Aspergillusterreus, Aspergillustamanii, Aspergillusniger etc.,[7]. The ASNase produced by Erwiniachrysanthemi and E.coli are presently used in the treatment of leukemia and lymphoma[8]In addition to medical application, ASNase is also used in food industrv.

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It is added to starchy foods before baking to prevent the formation of a carcinogenic compound, acrylamide. Acrylamide is formed during the heat treatment of starchy foods at high temperatures due to reaction between carbohydrates and L-asparagine. Acrylamide which is a neurotoxin is highly carcinogenic to both germinal and somatic cells. When ASNase is added to starchy foods before heat treatment it degrades L-asparagine preventing the formation of acrylamide[1, 4].In the present paper slaughterhouse, fish market and garden soil samples were processed in the laboratory to isolate and identify the ASNase producing bacteria.

2. MATERIALS AND METHODS

2.1 Sample collection

Soil samples from slaughterhouse, fish market and garden were selected for the study as these soils are protein rich (rich with organic material), a suitable condition for the occurrence of ASNase producing bacteria. The soil samples were collected in sterile polythene bags and preserved at 4^0C till further processing.

2.2 Medium

The M9 medium (Glucose - 3 g, $Na_2HPO_4.2H_2O$ - 6.0 g, KH_2PO_4 - 3.0 g, $MgSO_4.7H_2O$ - 0.5 g, NaCl - 0.5 g, $CaCl_2.2H_2O$ - 0.015g, L-asparagine - 3.0 g, and distilled water - 1 liter) incorporated with phenol red was used to isolate ASNase producing bacteria. Nystatin is added to medium to prevent the fungal growth. The initial pH of the medium was adjusted to 7.0[4]. The colour of M9 medium is yellow at pH 7.0. For the preparation of solid medium 20 g of agar is added to above medium. Soil solution of each soil sample was prepared by adding 10 g of each sample to 100 ml of sterile distilled water. Each soil solution is serially diluted and 0.2 ml of 10^{-7} dilution of each soil solution was spread on M9 agar medium incorporated with glucose, L-asparagine and phenol red and incubated for 48 hours at 30^0C. The bacteria which are capable of producing ASNase develop pink zone around their colonies[3, 4]. Uninoculated plate is maintained as control. The bacterial isolates of

slaughterhouse, fish market and garden soil samples positive for ASNase production were designated as A1, A2, A3…An, B1, B2, B3…Bn and C1, C2, C3…Cn respectively. The bacteria capable of producing ASNase were isolated and cultured on slants as pure cultures. Further each bacterial isolate is inoculated into M9 broth incorporated with glucose, L-asparagine and phenol red and incubated for 48 hours at 30^0C. The uninoculated broth tube serves as control. The colour of the bacterial broths in which ASNase is produced turns from to yellow to pink after incubation period due to the production of ASNase.

2.3 Production of ASNase and colour change

The phenol red exhibits yellow colour in medium at pH 7.0 and below and at pH above 7.0 pink or red colour. The M9 medium incorporated with glucose, L-asparagine and phenol red indicator appears yellow at pH 7.0. When bacteria positive for ASNase production were grown in M9 medium whose pH is 7.0 (appears yellow) they breakdown the L-asparagine and produce aspartic acid and ammonia. Due to the accumulation of ammonia which is a base the colour of the medium changes to yellow to pink. In solid medium pink zone is formed around the colonies due to accumulation of ammonia. Similarly in broth cultures the medium colour changes to yellow to pink.

2.4 Measurement of final pH in the culture broths and determination efficient ASNase producing bacteria

The changes in pH i.e., increase of pH (final pH) in the culture broths which turned yellow to pink was measured to determine the efficient ASNase producing bacterium. The culture broth in which more ASNase is produced exhibits maximum change in pH i.e., increase in pH due to more accumulation of alkali, ammonia which is produced due to the activity of ASNase[4].

2.5 Identification of ASNase producing bacterial isolates

Based on microscopic examination and biochemical tests the bacterial isolates were identified till their genus level. Gram staining, endospore staining and motility tests were performed for the bacteria. Then Indole test, Methyl red test, VogesProskauer test, Citrate utilization test, Phenyl alanine test, Hydrogen sulfide test, Mannitol salt test, Urease test, Oxidase test, Catalase test, Starch hydrolysis, Gelatin hydrolysis and Casein hydrolysis were conducted for the bacterial isolates to identify the bacteria till genus level[9].

3. RESULTS AND DISCUSSION

L-asparaginase produced by microbes can be employed for the treatment of cancer patients and it is also important for food industry especially in the preparation of baked foods to prevent the formation of a carcinogenic agent acrylamide. In the present study three types of soil samples viz., slaughterhouse, fish market and garden soil samples were processed in the laboratory using M9 medium supplemented with glucose, L-asparagine and phenol red to isolate and identify ASNase producing bacteria. Eight bacterial isolates were found to be positive for ASNase production which produced pink zones around their colonies in agar plates. The bacterial isolates positive for ASNase production were designated as A1 and A2 (from slaughterhouse soil), B1, B2, B3 and B4 (from fish market soil) and C1 and C2 (from garden soil). When these bacteria were grown in M9 broth supplemented with glucose, L-asparagine and phenol red the colour of the broth turned yellow to pink indicating their ability to produce ASNase (Figure-1). The final pH of all the culture broths was measured (Table-1). The culture broth of isolate B3 showed maximum change in pH and the final pH value was 8.70 which infer that it is the efficient producer of ASNase. Based on microscopic examination (Table-2) and biochemical tests all the bacteria were identified till genus (Table-3). The bacterial isolates of Slaughterhouse soil sample were identified as Serratia (A1) and Proteus (A2) species, bacterial isolates isolated from fish market soil sample were identified as Pseudomonas (B1), Staphylococcus (B2), Bacillus (B3) and Escherichia (B4) species and bacterial isolates of garden soil sample were identified as Bacillus (C1) and Acinetobacter species (C2). Kamble et al. (2012) characterized ASNase producing bacterial isolates occurring in water, farm and saline soils and identified them as Escherichia coli, Serratia, Pseudomonas, Bacillus, Aeromonas and Proteus species[3]. Vachani and Desai (2018) isolated ASNase positive bacteria from various soil samples and identified the bacterial isolates based on morphological and biochemical characteristics. The identified bacterial isolates include E.coli, Aerobacter, Pseudomonas, Bacillus, Vibrio, Xanthomonas, Serratia, Staphylococcus and Streptococcus[10]. Sahira et al. (2016) isolated ASNase producing Acinetobacterbaumannii from different blood and sputum samples to study its antibiofilm activity against certain pathogenic bacteria[11]. So far many researchers have been reported a wide range of ASNase producing bacterial species. These bacteria can be exploited in pharmaceutical and food industries.

Figure-1: *Culture broths (pink) positive for ASNase production and Control*

Table-1: *pH changes in bacterial culture broths due to the production ASNase*

S.No.	Bacterial isolate	Initial pH	Final pH
1.	A1	7.0	7.92
2.	A2	7.0	8.15
3.	B1	7.0	7.85
4.	B2	7.0	8.23
5.	B3	7.0	8.70
6.	B4	7.0	7.85
7.	C1	7.0	8.10
8.	C2	7.0	7.90

Table 2: Microscopic examination of bacterial isolates – Gram staining, Endospore staining and Motility test

S.No.	Bacterial isolate	Gram staining	Endospore staining	Motility test
1.	A1	Gram negative rod	Negative	Positive
2.	A2	Gram negative rod	Negative	Positive
3.	B1	Gram positive rods	Negative	Positive
4.	B2	Gram positive cocci	Negative	Negative
5.	B3	Gram positive rods	Positive	Positive
6.	B4	Gram negative rods	Negative	Positive
7.	C1	Gram positive rods	Positive	Positive
8.	C2	Gram negative rods	Negative	Negative

Table 3: Biochemical characterization of bacterial isolates to identify till their genus level

S.No.	Bacterial Isolate	Indole test	Methyl red test	VogesProskauerr test	Citrate test	Phenylalanine test	Hydrogen sulfide test	Mannitol test	Urease test	Oxidase test	Catalase test	Starch hydrolysis	Gelatin hydrolysis	Casein hydrolysis	Bacterium identified
1.	A1	-	-	+	+	-	-	+	+	-	+	-	+	+	Serratia
2.	A2	-	+	-	+	+	+	+	+	-	+	-	+	-	Proteus
3.	B1	-	-	-	+	-	-	-	-	+	+	-	-	+	Pseudomonas
4.	B2	-	+	+	+	-	-	+	+	-	+	-	+	-	Starphylococcus
5.	B3	-	+	-	-	-	-	-	-	-	+	+	+	+	Bacillus
6.	B4	+	+	-	-	-	-	+	-	+	+	-	-	-	Escherichia
7.	C1	-	+	-	-	-	-	-	-	-	+	+	+	+	Bacillus
8.	C2	-	-	-	+	-	-	+	-	-	+	-	+	-	Acinetobacter

4. CONCLUSION

ASNase is a medically important drug and it is also used in food industries in the preparation of baked foods. Various microbes serve as sources of ASNase. In the present study eight ASNase producing bacteria were isolated and identified till genus level. One of the bacterium (B3) which was identified as Bacillus species was determined as efficient producer of ASNase. These bacteria can be identified till their species level and the ASNase production by these bacteria can be optimized. Further, the efficient producers of ASNase among these bacterial isolates can be genetically improved to enhance the production of ASNase to exploit them commercially.

5. ACKNOWLEDGEMENTS

We are thankful to the Correspondent and Principal of Vivekananda Degree and PG College, Karimnagar for their cooperation and support during the completion of present work. Further we extend our thanks to Administrative officer and Head, Department of Computer Science, Vivekananda Degree and PG College for permitting us to use computer lab during the preparation of paper.

REFERENCES

[1]. M. Ushakiranmayi, M. Vijayalaxmi, S.R.K.G. Venkat, B.R Satish and P. Sudhakar,"Extracelluar L-Asparaginase from Streptomyces labedae VSM-6: Isolation, Production and Optimization of Cultural Conditions Using RSM," Pharmacognosy Journal, vol. 9, no.6, pp. 932-941, Nov/Dec 2017, doi: 10.5530/pj.2017.6.146

[2]. G. Saeed, G. Soudabeh, A. Nazemi and N.M. Miri,"Isolation and Characterization of a Novel Bacillus sp. Strain that Produces L-asparaginase, and Antileukemic Drug," Asian Journal of Biological Sciences, vol. 6, no. 2, pp. 106-115, 2013, doi:10.3923/ajbs.2013.106.11

[3]. K..D. Kamble, P.R Bidwe, V.Y. Muley, L.H. Kamble, D.G. Bhadange, and M. Musaddiq. "Characterization of L-Asparaginase producing bacteria from Water, Farm and Saline Soil," Bioscience Discovery, vol.3, no. 1, pp. 116-119, Jan2012

[4]. S. Yogendra and S.K. Srivastava"Isolation and Characterization of Microorganisms capable of producing antineoplastic drug, L-asparaginase," International Journal of Biological and Medical Research, vol. 3 no. 4, pp. 2548-2554, 2012

[5]. M.R. Bhat, J.S. Nair andT Marar, "Isolation and Identification of L-Asparaginase Producing Salinococcus sp. M KJ997975 from Soil Microbial Flora," International Journal of Pharmaceutical Sciences and Research, vol.6,pp. 8, 3599-3605, Aug 2015, doi: 10.13040/IJPSR.0975-8232.6(8).3599-05

[6]. A. Jayakumar, P.A. Balakrishna and H. Kumarapillai."Sequencing and Characterization of L-asparaginase (ansB) gene of Bacillus megatherium isolated from Western Ghats, Kerala, India," International Journal of Current Microbiology and Applied Sciences, vol. 4, no. 6, pp. 753-760, 2015.

[7]. M.D. Osama, F.E. Mohamed, A..M and Ibrahim,"Isolation, Screening and optimization of L-asparaginase producing bacterial strains inhabiting agricultural soils" Bioscience Research, vol. 15, no. 3, pp. 2802-2812, Sep 2018

[8]. S.A. Alrumman, Y.S. Mostafa, A.A. Kholood, M.Y. Alfaifi, T.H. Taha and S.E. Elbehairi,"Production of Anticancer Activity of an L-Asparaginase from Bacillus licheniformis Isolated from the Red Sea, Saudi Arabia" Scientific Reports, vo.9, 1-14, Mar 2019, https://doi.org/10.1038/s41598-019-40512-x

[9]. A. Ravi and R.P. Praveen."Isolation, Biochemical Characterization and Identification of Oil Degrading Bacteria Occurring in Oil Contaminated Sites of Mechanical Workshops," International Journal of Pure and Applied Bioscience, vol. 4, no. 6, pp. 102-106, Dec 2016, http://dx.doi.org/10.18782/2320-7051.2418

[10]. B. Desai and J. Vachani,"Isolation, Identification and Production of L-asparaginase producing bacteria from Soil" BMR Microbiology, vol. 4 no. 1, pp. 1-6, Apr2018

[11]. N.M. Sahira, M.S.A. Israa, N.M.A. Alaa, H.H. Nadheema, S.D. Ahmed, T.E. Ban, N.M. Sraa and A.A. Suhad,"Extraction and Purification of L-Asparaginase produced by Acinetobacterbaumannii and their Antibiofilm Activity against some Pathogenic Bacteria," The International Journal of Biotechnology, vol. 5 no. 1, pp. 7-14, 2016, doi: 10.18488/journal.57/2016.5.1/57.1.7.14