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SCREENING, OPTIMIZATION OF MEDIUM AND SOLID STATE FERMENTATION FOR L-ASPARAGINASE PRODUCTION

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ABSTRACT
The enzyme L-asparaginase has been proved to be efficient in treatment of Acute Lymphoblastic Leukemia [ALL] and Lymphosarcomas. The present paper discusses the studies carried out for the production of this enzyme from bacillus species. By study obtained optimization of medium, solid state fermentation by using cotton cake and wheat bran and submerged fermentation using distillery waste. a M2 modified medium has optimal L-asparaginase production. The enzyme activity (4.58EU/mL) markedly increases in M2 modified medium (62.33EU/mL), solid state fermentation has shows desired enzyme production in cotton cake (68.00EU/mL), and wheat bran(22.91EU/mL).in distillery waste carried submerged fermentation for L-asparaginase production.

KEYWORDS: L-asparaginase production, solid state fermentation, optimization of medium *Bacillus species*.

INTRODUCTION

L-Asparaginase (E. C. 3. 5. 1. 1) is present in many animal tissues, bacteria, plants, and in the serum of certain rodents, but not in humans[1] Mukherjee *et al.* (2000) studied nutritional and oxygen requirements by Enterobacter aerogenes for production of L-asparaginase. Microbial enzymes are preferred over plant or animal enzymes due to their economic production, consistency, ease of process modification and optimization. They are relatively more stable than corresponding enzymes derived from plants or animals. Further, they provide a greater diversity of catalytic activities. The enzyme is produced by diverse kind of microorganisms that include *E. coli* [2], *Erwinia cartovor*[3,4], *Enterobacter aerogenes*[5], *Candida utilis* [6], *Staphylococcus aureus*[7], , *Thermus thermophilus*[8], and *Pisum sativum*[9] *Aspergillus tamari, Aspergillus terreus*[10,11] *and Pseudomonas stutzeri*[12]. L-asparaginase (L-asparagine amidohydrolase (EC 3.5.1.1) is used in the treatment of acute lymphoblastic leukemia and It is also used for the treatment of pancreatic carcinoma and bovine lymphomosarcoma[13]. L-asparaginase (E.C. 3.5.1.1) is an enzyme which catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia [14]

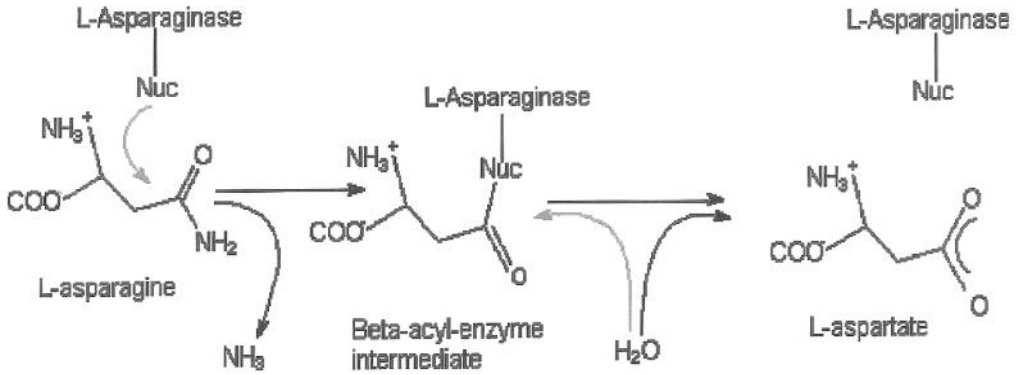

FIGURE 1. Schematic illustration of the reaction mechanism of L-asparaginase (Hill *et al*., 1967)

Cancer cells use L-asparagine as source of nitrogen for their metabolism, but these cells are unable to synthesize this amino acid, and mainly depend on the L-asparagine from circulation. Clinical trials indicate that this L-Asperginase enzyme is also a promising agent in treating some forms of neoplastic cell disease in man. It catalyses the conversion of L-asparagine to L-aspartate and ammonium, and this catalytic reaction is essentially irreversible under physiological conditions. This clinical action of enzyme is attributed to the reduction of L-asparagine. Since tumor cells are unable to synthesize L-aspargine they are selectively killed by L-asparagine deprivation[15]. The submerged fermentation is admirably suited for a flexible operation. It is possible to introduce certain process accelerators or nutrients at desired and convenient time intervals. The majority of industries have

adopted submerged fermentation process. Solid-state fermentation is a very effective technique as the yield of the product is many times higher when compared to that in SMF, and it also offers many other advantages. In cotton cake and wheat bran used for enzyme production by Solid-state fermentation using *Bacillus sp.* In present work we used both submerged and solid state fermentation process for the production of L-Asperginase, solid state fermentation was carried out by using cotton oil cake.

MATERIALS & METHODS

The water samples collected from Lonar Lake, marine water (Alibag) and Dhobighat were used for isolation of L-Asperginase producing microorganisms.

Screening of Microorganisms for L-asparaginase production

The isolated Microorganisms were screened for L-Asparaginase production by using "A rapid plate assay" For that M9 medium was used with L-Aspergine as substrate and phenol red as pH indicator[16].Colonies showing pink color zones around them were further used for secondary screening.

Secondary screening for L-Asparaginase producers was performed in shake flask culture[17] Individual isolates selected in primary screening were inoculated in M3 medium [18] and kept at 37^0c for 72 hours. After incubation these were checked for the L-Asparaginase activity and strain showing highest activity (A26) was used for production of L-Asparaginase.

Submerged fermentation

50ml of M9 medium was autoclaved and inoculated with loopful culture of A26.The inoculum media was incubated at 37^0C for 24 hrs in rotary incubator shaker at 120 rpm.

50ml of M9 medium was taken in 250 ml capacity Erlenmeyer flask. Medium was autoclaved at 121^0C for 15 min. After sterilization 1ml inoculum was transferred to it. The flask was then incubated at 37^0c for 72 hours on rotary incubator after completion of the fermentation; the fermented broth was centrifuged at 8000rpm for 10 min. The supernatant was collected and used for enzyme assay.

Media optimization for submerged fermentation

Sr. No.	MEDIA	COMPOSITION OF MEDIA (g/l)
1.	Nutrient medium M1 (Modified)	Glucose 1.0;K_2HPO_4 1.0; Yeast extract 5.0; Tryptone 5.0, L-Aspargine 0.1% (W/V); pH7.0
2.	Nutrient medium M2.	Fructose 1.0;K2HPO4 1.0; Yeast extract 5.0; Tryptone 5.0; pH7.0
3.	Nutrient medium M2 (Modified)	Fructose 1.0;K2HPO4 1.0; Yeast extract 5.0; Tryptone 5.0, L-Aspargine 0.1% (W/V pH7.0
4.	Nutrient medium M3 (Modified)	Lactose 1.0;K2HPO4 1.0; Yeast extract 5.0; Tryptone 5.0; L-Aspargine 0.1% (W/V; pH7.0
5.	Nutrient medium M4 (Modified)	Sodium formate(100 mM); Sodium fumarate (100 mM); Yeastextract 0.5%; L-Aspargine 0.1% (W/V);pH .6.8
6.	Nutrient medium M5	Peptone 5.0;NaCl 5.0; Yeast extract 1.5%; beef extract 1.5;pH 7.2

The various types of media were used for L-Asparaginase production by submerged fermentation[18]. The media modified M_1, M2, modified M_2, M_3 modified M_3, modified M_4, M_5 were used for media optimization. The media named with prefix "modified" refers to the media components with 0.1% aspargine. In these media the carbon sources and nitrogen sources were varied. These media were inoculated with inoculum of strain A26 and kept at 37^0C on shaking incubator for 72 hours. After incubation the fermented broth was assayed to determine L-asparaginase activity.

Use of distillery waste as fermentation medium

The spent wash (distillery waste) was also used for L-asparginase production. The distillers waste collected from distillery and diluted to 1% by using distilled water. Such distillery waste was directly as medium. The inoculum of A26 strain was added and kept for fermentation on rotary shaker at 37^0C for 72 hours. The fermentation using distillery waste with L-asparagine (1%) was also carried out.

Solid state fermentation

For production of L- asparaginase using solid state fermentation, the agricultural waste such as wheat bran and cotton oil cake was used. Wheat bran and cotton oil cake were grinded separately and used directly as medium after autoclaving at 121^0C for 20 min.10 g agricultural waste (wheat bran/cotton oil cake) was added with 5ml inoculum and 5ml tris buffer (0.1 M) of pH 7 was added to moisten the medium .The same media with 0.1% asparagine was also used for fermentation. After inoculation the media were kept for incubation at 37^0c for 72hours. After fermentation crude enzyme was recovered and assayed to determine L-asparaginase activity.

Enzyme assay

The enzyme assay for L-asparaginase was performed by method given by Mashburn, L.T.[19]. The assay method includes spectrophotometric stop rate determination. -The principle of assay is that L-asparagin in presence of asparaginase forms L-asparatate and ammonia (NH_3). The liberated ammonia can be estimated by using Nesselers reagent. 1µmole of ammonia liberated refers to the 1 international unit (IU) of L-asparaginase. (Include the procedure of assay with the concentration of reagents used). Immediately mix by inversion and after 1 min. Absorbance was read at 436nm for test and blank. Graph of optical density verses ammonia concentration (note that 1M of ammonium sulphate corresponds to 2M ammonia therefore a 6 mM ammonium sulphate standard is equivalent to

Identification of bacteria

For identification of bacteria the Grams staining, flagella staining, endospore staining was performed. The colony characters also studied.

RESULTS & DISCUSSION

About 63 microorganisms were isolated from the water samples collected from Lonar Lake and marine water (Alibag). The Jayaramu *et al.* (2011) isolated L-asparginase producing bacteria from water sample.

Screening of microorganism

The isolated organisms were screened for L-Asparaginase production by rapid plate assay. Among 63 isolates, 8 organisms show larger zones of asparginase production and covered whole plates within 72 hrs. These 8 isolates showing larger zones *i.e.* better Asparaginase production were processed for secondary screening and shows result as shown in Fig.1. Among these 8 Asparaginase producers isolate A_{26} was selected due to its high enzyme producing ability in terms of EU/mL. Asparginase producing ability of A_{26} confirmed by growing the culture on different media variants (Asparagine$^+$ dye$^+$, Asparagine$^-$ dye$^+$ and Asparagine$^+$ dye$^-$, $NaNO_3$ + dye) and shows the pink zone on plate containing Asparagine as nitrogen source and dye (phenol red) as indicator for Asparaginase production. The plate with asparagine as nitrogen source and without dye doesn't show pink zone, it rejects any possibility of pigment production. The plate with $NaNO_3$ as nitrogen source and dye also doesn't show pink zone. From these observations it can be concluded that pink zone was due to asparginase production only shown in fig 2. The efficiency of A_{26} for Asparaginase production also assessed by growing the culture in shake flask shown in fig. 2 and shows that the inoculated broth turned dark pink in color as compared to control. (Jayaramu *et al*., 2010). The similar type of screening was performed by Hosmani *et al*. (2011).

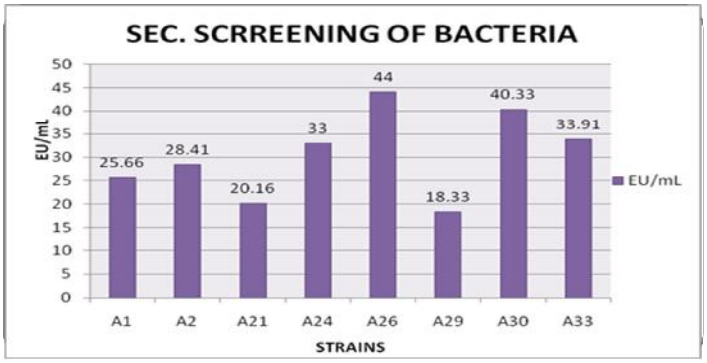

FIGURE-1 Secondary screening of Asparginase producing bacteria

FIGURE 2. Rapid plate assay for screening of L-asparaginase Production.

FIGURE 3: Rapid flask assay for screening of L-asparaginase Production

Submerged fermentation

Submerged fermentation was studied using M9 medium. After 72 hrs produced asparginase was measured in terms of EU/ml. Presence of 14.25 U and 52.25 EU/ml of asparginase was found in uncentrifuged and centrifuged fermentation broth. By submerged fermentation the fermentation parameters can be controlled to get maximum yield. The similar types of results were found by Radhika Tippani (2012), and A. R. Soniyambi (2011).

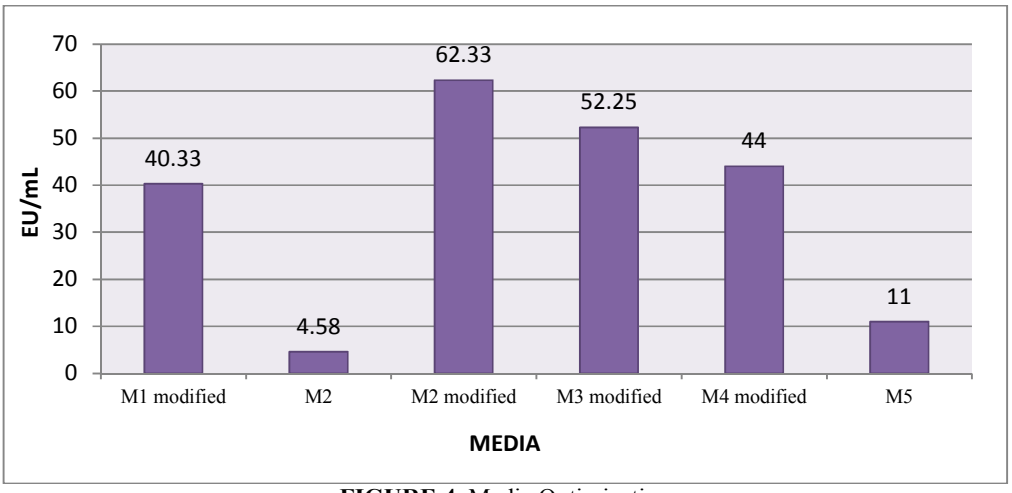

FIGURE 4. Media Optimization

Fig.4 shown optimization of medium among the six media M_2 modified medium shows asparginase production activity in terms of EU/ml. The higher production of L-Asparaginase was found in media containing sucrose as carbon source. Mohana Priya, P. (2011) and K. Krishna Raju Patro (2011) also found that higher asparginase was produced in media containing sucrose as carbon source.

Use of distillery waste for production of L-asparaginase

Distillery waste i.e. waste resulting from distilleries in the form of spent wash can be used in production of L-Asparaginase. The desired strain of bacteria unable to grow in distillery waste as it is. To grow bacteria in distillery waste it was diluted to 1%. The enzyme produced in distillery waste (1%) was found to be 3.66 EU/mL. When the distillery waste (1%) was added with sucrose as an additional carbon source then L-Asparaginase produced was 1.21 EU/mL. When the distillery waste (1%) was added with yeast extract as additional nitrogen source then L-Asparaginase produced was 8.54 EU/mL. From this it was found that the distillery waste (1%) with added yeast extract produced comparatively higher amount of L-Asparaginase.

TABLE 1: Distillery waste for production of L-Asparaginase

Sl.No.	Medium	EU/ml
1	Distillery waste (1%)	3.66
2	Distillery waste (1%)+ Sucrose	1.21
3	Distillery waste (1%)+ Yeast extract	8.54

Solid state fermentation

In this, the enzyme recovered by using buffer and this crude enzyme shows enzyme activity as follows. The enzyme activity using two different substrates was as follows.

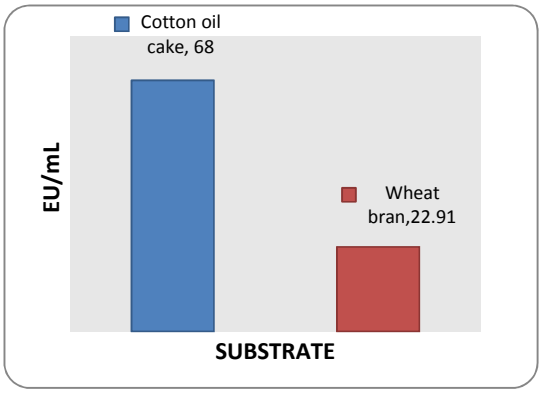

FIGURE 5. Solid state fermentation

From above fig 5 this yield of L-Asparaginase, it was found that cotton oil cake shows high productivity as compared to the wheat bran. The similar types of results were found by Siddalingeshwara K. G. (2011) and Radhika Tippani (2012) by Solid state fermentation. As we got the high enzyme units using cotton oil cake, then performed experiment in triplicates the mean EU/mL found was 61.08.

Identification of bacteria

The colony characters, grams nature of bacteria, endospore forming ability of bacteria and presence of flagella was determined. It was found that the bacteria were gram positive rods for further identification of bacteria the culture sent to the NCCS Pune for 16s rRNA sequencing. 16S ribosomal RNA gene, partial sequence shows that the bacterium is *Bacillus sp.*

CONCLUSION

From collected water samples 63 bacteria were isolated. The primary screening of isolates was done and among 8 primary screened bacteria A26 and A30 shows good production of L-Asparaginase during secondary screening. Comparatively A26 shows more L-Asparaginase production hence used for further study. In submerged fermentation A26 shows good production of L-Asparaginase. The optimum incubation time for production of L-Asparaginase was found to be 72 hours. In media optimization modified M2 medium shows higher production of L-Asparaginase[19]. The distillery waste also used in submerged fermentation for production of L-Asparaginase but shows lower yield. The optimum pH for enzyme activity was 8.6 and optimum temperature was 50^0C. In solid state fermentation using cotton oil cake as substrate shows higher production of L-Asparaginase[11]. Partial purification of L-Asparaginase was carried out and which results in 6.21 fold increase in enzyme activity.

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