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**Research Article**

# OPTIMIZATION OF ASPARAGINASE ENZYME USING NATURAL SUBSTRATES BY SSF FROM MORAXELLA sp ISOLATED FROM CLAY FACTORY WASTE WATER AND EVALUATION OF ITS PHARMACEUTICAL APPLICATIONS

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**ABSTRACT**

L-asparaginase plays a vital role in medical application, particularly in treatment of acute lymphoblastic leukemia as an effective anti-tumor agent. The present study aims to isolate and identify asparaginase producing microorganism present in clay factory waste water and to evaluate the biological activities such as antimicrobial, hemolytic and anticancer activity exhibited by the bacterial strains. This study also involves optimization of L-asparaginase production under solid state fermentation using agro by products such as coconut oil cake, cotton seed oil cake and groundnut oil cake. The present study reveals that *Moraxella* sp isolated from clay factory waste water showed the highest asparaginase and hemolytic activity. On optimization maximum asparaginase enzyme activity was shown by coconut oil cake. Present study also revealed that the asparaginase enzyme collected from species exhibited potent cytotoxicity against A549 cell line.



## INTRODUCTION

Nature acts as a prominent reservoir for new and novel therapeutics. Enzymes are biological polymers that catalyse the multiple dynamic processes which make life processes possible. In recent years enzymes have gained great importance in clinical research. Enzymes play central role in health and disease because they are the determinants of the rate at which physiologic event take place. Asparaginase is one of them which are widely present in nature. Asparaginase catalyses the hydrolysis of asparagine into aspartic acid and ammonia. Asparaginase has received increased attention in the recent years for its anticarcinogenic potential (Manna *et al.*, 1995). The therapeutic enzyme is mainly used in treatment of different forms of cancer. Asparaginase has been a clinically satisfactory antitumor agent for the valuable treatment of acute lymphoblastic leukemia and lymph sarcoma (Savitri *et al.*, 2003).

Solid state fermentation (SSF) is suitable for the production of enzyme by using natural substrate because they mimic the condition under which the microbe grows natured. Solid state fermentation (SSF) has emerged as an alternate cost- effective process for bioproducts production by utilizing the agrowastes and its byproducts. It has been employed for production of various microbial metabolites such as enzymes, organic acids and antibiotics. In this context, an effort has been initiated to evaluate the antimicrobial, hemolytic, anticancer activity against A549 cell line.

## MATERIALS AND METHODS

### *Sample Collection and Isolation of bacterial cultures*

The water samples were collected in the sterilized sampling bottles from Clay factory, Veli, Trivandrum. The collected samples were preserved in refrigerator for further use. The isolation of bacteria from water sample was carried out by serial dilution method (Walksman& Reilly, 1945) and spread plate technique (Shiriling and Gottleib, 1966). Characterization of bacterial strains were performed based on the method followed by Bergy's Manual of determinative bacteriology (Bergy *et al.*, 2004).

### *Qualitative assay for production of L-asparaginase by isolated bacteria*

Agar diffusion technique was used for qualitative assay of L-asparginase by isolated bacterial cultures. Modified Czapek

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Dox's Medium (pH 6.8) was used as an assay medium. Sterilized medium (10 ml) was distributed in the presterilized culture tubes to prepare stabs. After that a loopful culture of each isolate was inoculated on the surface of solidified stabs and incubated at 37°C for 24 to 48 hours. Uninoculated stab was regarded as a negative control. Stabs were examined for change in color of medium from yellowish to pink.

***Production of L-asparaginase***

The secondary screening of bacterial isolates for L-asparaginase production was done on modified M9 broth medium (pH 7) supplemented with L-asparagine (1%w/v), which was inoculated with bacterial cultures and incubated for 24 hours at 37°C; 35 ml of medium was taken in a conical flask of 150 ml and inoculated with 1 ml culture of bacteria and incubated at 37°C for 24 hours. After completion of the incubation period, culture broth was centrifuged at 5,000 rpm for 20 min at 4°C. To assay intracellular L- asparaginase enzyme's activity, cell pellet was suspended in 15 ml of 0.1M Tris-HCl buffer (pH 7.4) and sonicated using ultra-sonicator for 15 min at 4°C. Cell debris was removed by centrifuging at 4°C for 10min at 10,000 rpm (Cedar *et al*., 1968).

***Quantitative assay for production of L-asparaginase by isolated bacterial cultures***

The quantitative estimation of enzyme activity was done with selected culture isolates. Asparaginase activity was measured by method of Mashburn & Wriston, 1963. The rate of hydrolysis of L-asparagine was determined by measuring the release of ammonia using Nessler's reaction.

***Optimization of L- asparaginase production under solid state fermentation using natural substrate***

Optimization of L-asparaginase production was carried out in three natural substrates Coconut oil cake (COC), Cottonseed oil cake (CSOC) and Groundnut oil cake (GOC) were purchased from the local market. In this the isolated bacterial culture was maintained at $37^0$C on Luria Bertani (LB) agar slants. Inoculum was prepared by adding a loopful of bacterial colonies in 250ml Erlenmeyer flasks containing 100ml of LB broth and incubated in a rotary shaker at 37 $^0$ C for 24 hours. Then 2ml of overnight grown culture was inoculated into the Erlenmeyer flask containing 10 g of the respective substrate. The flasks were incubated at $37^0$C in incubator and the contents mixed at regular intervals. Fermented samples were taken and assayed for enzyme activity (Sangitha Ghosh *et al*., 2004).

Extraction of crude enzyme was done by adding 100ml of sodium phosphate buffer (pH 7) to the fermented substrate, and kept in rotary shaker for 45 min. One ml of the extract was transferred to the eppendorf tube and centrifuged at 10000 rpm for 10 minutes. L-asparaginase activity was determined by measuring the amount of ammonia released by nesslerization according to the method described by (Wriston and Yellin., 1973). 0.2 ml of cell free supernatant was mixed with 0.8 ml of 0.1 M sodium borate buffer (pH 8.5) and 1 ml of 0.04 M L-asparagine and incubated for 10 min. 0.5 ml of 15% TCA was added to stop the reaction and again centrifuged at 10000 rpm for 10 minutes. 0.2 ml of the supernatant was taken in a test tube in which 3.6 ml distilled water and 0.2 ml Nessler's reagent were added. The optical density was measured at 480 nm in UV spectrophotometer. One Unit (U) of L-asparaginase is defined as the amount of enzyme required to liberate one µ mol of ammonia per min at 37°C.

***Purification of L- asparaginase***

L- Asparaginase was purified from the culture filtrate. The enzyme was partially purified by ammonium sulphate precipitation (39%). The mixture was left overnight at $4^0$C, followed by centrifugation at 6000 rpm at $4^0$C for 15 minutes. The precipitate was dissolved in 0.1 M phosphate buffer (pH 7) for further purification. 5 ml of the partially purified enzyme was dialyzed against 0.01 M Tris buffer at $4^0$C. The partially purified sample was assayed for enzyme activity and protein content. Partially purified enzyme was loaded into a silica gel column washed with distilled water and then with Tris buffer (0.5M) and left overnight. The partially purified enzyme is added through the column and fractions were collected. The pooled samples from the column were used for further analysis (Manna *et al*., 1995).

***Determination of antimicrobial activity***

The antimicrobial susceptibility testing was done by Kirby Bauer Disc diffusion method (Bauer *et al*, 1996) which allows the rapid determination of the efficiency of bacterial isolates against various pathogenic bacteria's. In agar diffusion method a filter disk impregnated with an antibiotic is applied to the surface of an agar plate containing the organism to be tested and the plate is incubated at 37°C for 24-48 hours. As the substance diffuses from the filter paper into the agar, the concentration decreases as a function of the square of the distance of diffusion. At some particular distance from each disk, the antibiotic is diluted to the point that it no longer inhibits microbial growth. The effectiveness of a particular antibiotic is shown by the presence of growth-inhibition zones. These zones of inhibition (ZOIs) appear as clear areas surrounding the disk from which the substances with antimicrobial activity diffused.

***Hemolytic activity***

Hemolytic activity was tested on blood agar plates. Plates were prepared with blood agar and it was sterilized by autoclaving. Human blood (5%) was prepared and prior to pouring, blood was added. Then the sample was loaded on agar plate and incubated at $37^0$ C for 48 hours (Carillo *et al*., 1996).

***Anticancer activity***

***MTT assay***

The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD (P) H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color (Arung *et al*., 2000). The absorbance values were measured at a wavelength of 570 nm (Laura *et al*., 2004).The percentage of growth inhibition was calculated using the formula:

$$\% \text{ viability} = \frac{\text{Mean OD Samples x } 100}{\text{Mean OD of control group}}$$

## RESULTS

The present study mainly focus on the isolation and identification of bacteria from Clay factory waste water, production of L-asparaginase, optimization and its purification, antimicrobial screening, hemolytic activity, and anticancer activity.

### *Isolation and identification of microorganism from water sample*

Totally 9 bacterial strains were isolated from clay factory waste water and named as S1 to S9. Based on the Bergy's manual of Determinative Bacteriology, (2000) the isolated bacteria's were identified as *E. coli* (S1), *Pseudomonas* sp. (S2), *Enerobacter*sp. (S3 & S5), *Acetobacter* sp. (S4 & S9), *Streptococcus* sp. (S6 & S8) and *Moraxella* sp. (S7).

### *Qualitative assay of L- asparaginase by isolated cultures*

Among the 9 isolates asparaginase activity was shown by bacteria's *Pseudomonas* sp. (S2), *Moraxella* sp. (S7) and *Acetobacter* sp. (S4&S9)

### *Quantitative assay of L-asparaginase from isolated bacterial strains*

The quantitative estimation of enzyme activity was done with selected culture isolates *Pseudomonas* sp., *Moraxella* sp. and *Acetobacter* sp. Among the isolates *Moraxella* sp (0.283) showed the high asparaginase activity than the other two isolates *Pseudomonas* sp (0.251) and *Acetobacter* sp. (0.243).

### *Optimization of L- asparaginase production under solid state fermentation using natural substrate*

Optimization of L- asparaginase was carried out by using Coconut oil cake (COC), Cotton seed oil cake (CSOC) and Groundnut oil cake (GOC) as carbon source for L-asparaginase production by *Moraxella* sp. All the three substrates showed positive results for the production of the enzyme.COC showed maximum enzyme activity (2.302) as compared to CSOC (0.520) and GOC (0.108). In this study coconut oil cake (COC) showed better results than the others Cotton seed oil cake (CSOC) and Groundnut seed oil cake (GOC).

**Table 1** Optimization of L- asparaginase production from *Moraxella* Sp

| Substrates | Substrate concentration(gm) | L-Asparaginase Activity(U/gds) |
|---|---|---|
| Coconut oil cake | 10 | 2.302 |
| Groundnut oil cake | 10 | 0.108 |
| Cotton seed oil cake | 10 | 0.520 |

**Fig 1** Optimization of L-Asparaginase production

### *Purification of asparaginase enzyme*

L-asparaginase was purified by ammonium sulphate precipitation (39%) and fractions were purified by dialysis. The purified fractions were separated by column chromatography.

### *Antimicrobial screening of bacterial enzyme*

The bacterial isolates were screened for antibacterial activity against *Salmonella typhi*, *Enterobacter aerogens*, *Proteus vulgaris*, *Escherichia coli* and *Shigella sonnei* by Kirby Bauer agar well diffusion method (Bauer *et al.*,). *E.coli* showed highest activity against *Proteus vulgaris* (0.8) than *Shigella sonnei* and *Salmonella typhi* and no activity against *Escherichia .coli* and *Enterobacter aerogens*. *Pseudomonas* sp showed highest activity against *Shigella sonnei* (0.9) and no activity against *Salmonella typhi*. *Enterobacter* sp. showed highest activity against *Escherichia .coli* and *Proteus vulgaris* (0.9) and lowest activity against *Salmonella typhi* (0.6).

*Acetobacter* sp. showed highest activity against *Enterobacter aerogens* (1.1) and no activity against *Proteus vulgaris*. *Streptococcus* sp. showed highest activity against *Proteus vulgaris* and no activity against *Escherichia .coli* and *Shigella sonnei*. *Moraxella* sp. showed highest activity against *Escherichia .coli*, *Shigella sonnei* and *Salmonella typhi*. The isolated enzyme only showed activity against *Enterobacter aerogens*. The results are tabulated:

**Table 2** Antimicrobial Screening of Isolated Bacterial Strains

| | S1 | S2 | S3 | S4 | S6 | S7 | Enzyme |
|---|---|---|---|---|---|---|---|
| **Name of bacteria** | **Zone of inhibition in cm** | | | | | | |
| *Escherichia coli* | - | 0.8 | 0.9 | 0.7 | - | 0.8 | - |
| *Enterobacter aerogenes* | - | 0.8 | 0.8 | 1.1 | 0.8 | 0.7 | 0.9 |
| *Proteus vulgaris* | 0.8 | 0.7 | 0.9 | - | 1.0 | 0.7 | - |
| *Shigella sonnei* | 0.7 | 0.9 | 0.8 | 0.9 | - | 0.8 | - |
| *Salmonella typhi* | 0.7 | - | 0.6 | 1.0 | 0.9 | 0.8 | - |

### *Hemolytic Activity*

The highly active strains of *Acetobacter* sp, *Moraxella* sp and *Streptococcus* sp was placed on the blood agar plates and incubated for 24- 48 hours at $37^0$ C. Among the isolates *Moraxella* sp (3.4mm) showed highest hemolytic activity so this strain was subjected to anticancer activity.

**Table 3** Hemolytic activity of isolated bacterial strains

| Bacterial isolates | Hemolytic activity(mm) |
|---|---|
| *Acetobacter* sp | 2 |
| *Moraxella* sp | 3.4 |
| *Streptococcus* sp | 2.2 |

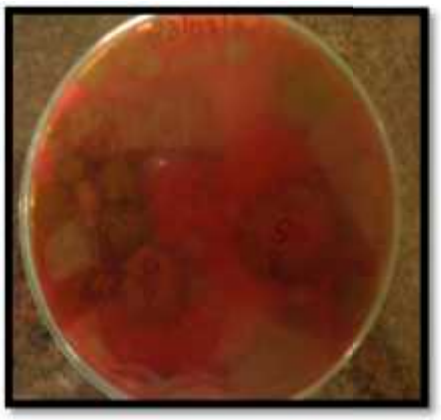

**Fig 2** Hemolytic activity of bacterial isolates

### *Anticancer activity*

### *MTT assay*

Invitro cytotoxicity assays are a potentially useful tool in the study of toxic compounds of complex mixtures. In the present study it showed that the asparaginase enzyme collected from species exhibited potent cytotoxicity against A549 cell line. Cells were treated with asparaginase enzyme at concentration of 100µl and then the percentage of cell viability was analyzed. The results of MTT assay showed a dose dependent inhibition of growth of A549 cell line.

**Table 4** Anticancer Activity of Isolated Enzyme

| Sample volume (µl) | Average OD at 540nm | Percentage of cell Viability (%) |
|---|---|---|
| **Control** | 0.8889 | |
| 6.25 | 0.7755 | 87.24266 |
| 12.5 | 0.711 | 79.9865 |
| 25 | 0.6302 | 70.89661 |
| 50 | 0.5599 | 62.98796 |
| 100 | 0.3917 | 44.0657 |

## DISCUSSION

In recent years enzymes have gained great importance in clinical research. Enzymes play central role in health and disease because they are the determinants of the rate at which physiologic event take place. Asparaginases are enzymes that occur naturally and are distributed among living organisms including animals, plants and microbes (Mukherjee *et al*, 2000; Siechiechowcicz & Ireland, 1989; Clementi, 1922). Discovery of L-asparaginase, as a medicinal agent for treatment of cancer, was made in1922. The *E.coli* and *Erwinia* enzymes were isolated, purified and experimentally used as an anti-leukaemic agent in human patients (Story *et al*, 1993).

The importance of micro-organisms as L-asparaginase sources has been focused since the time it was obtained from *E. coli* and its anti-neoplastic activity demonstrated in guinea pig serum (Schawartz *et al*, 1966; Boyse *et al*, 1967). *Erwinia caratovira, corynebacterium glutamicum, Bacillus* sp, *Pseudomonas strutzeri and E.coli* are most commonly used microorganisms for the production of asparaginase. *Moraxella* is a genus of Gram-negativebacteria the Moraxellaceae family. Only there are a little reference on L asparaginase activity in *Moraxella* sp. Hence the present study is an attempt to denote the L asparaginase activity in certain bacteria isolated from clay factory waste water. Water samples were collected from English India clay limited in veli. Various bacterial strains were isolated among which *Pseudomonas* sp, *Acetobacter* sp and *Moraxella* sp showed asparaginase activity. Of all these *Moraxella* sp was selected for the experimental studies because it exhibited elevated activity of this enzyme. Then the isolated asparaginases were subjected to anticancer activity against A549 cell line. The results of MTT assay showed a dose dependent inhibition of growth of A549 cell line.

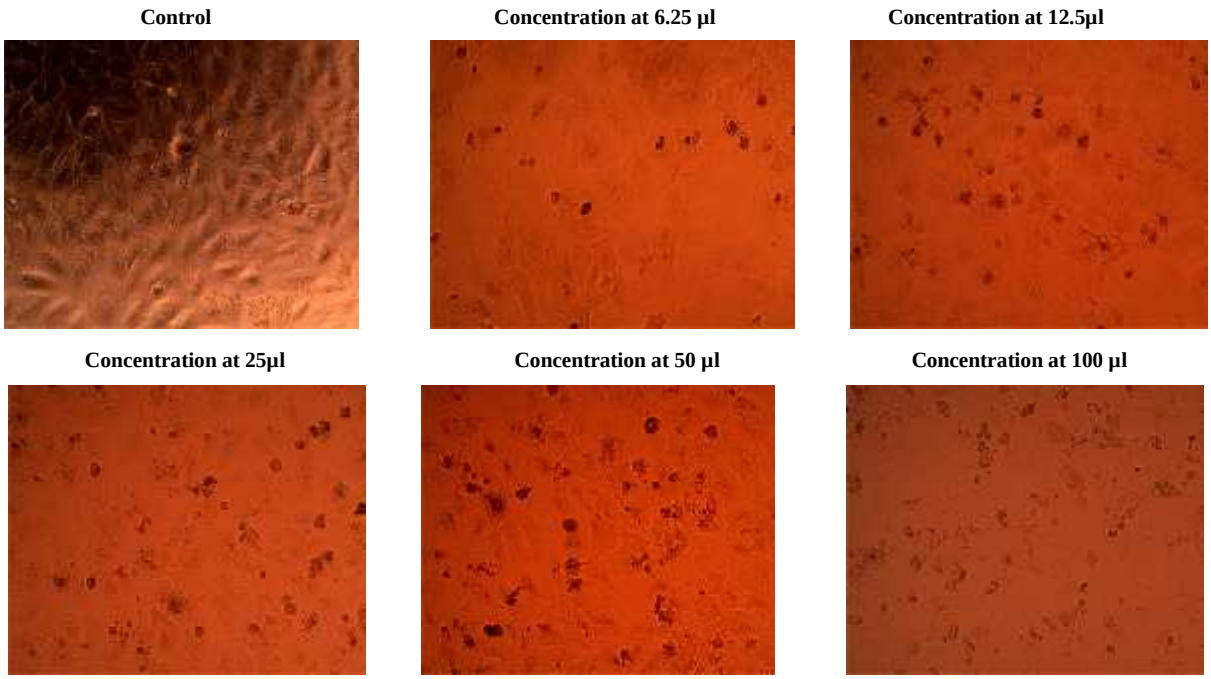


**Fig 2** Anticancer activity of Enzyme extract on A549 cell line

Antimicrobial activity and hemolytic activity were also evaluated. Antimicrobial study revealed that isolated asparaginase enzyme showed activity against *Enterobacter aerogens.*Among the bacterial isolates *Moraxella* sp showed highest hemolytic activity. Although L-asparaginase inhibits tumor cell growth, studies of this phenomenon have been limited, usually because sufficient quantities of the enzyme were not available. Therefore alternative methods are used for production of asparaginase enzyme. For large scale production of asparaginase enzyme optimization of asparaginase enzyme was performed by using natural substrate under solid state fermentation. In this study Coconut oil cake (COC), Cottonseed oil cake (CSOC) and Groundnut oil cake (GOC) were used as carbon source for L- asparaginase production by *Moraxella* sp. All the three substrates showed positive results for the production of the enzyme with yields varying .Among which Coconut oil cake (COC) showed maximum enzyme activity. The results of the present study also suggest that isolated L-asparaginase may prove to be a promising agent and requires further investigation of its potential anti-leukemic activity.

## CONCLUSION

Microorganisms have become a rich source of several enzymes that may have some unique properties of interest for both basic research and pharmaceutical applications. Asparaginase has been a clinically satisfactory antitumor agent for the valuable treatment of acute lymphoblastic leukemia and lymph sarcoma. In the present study the isolated microorganisms present in the clay factory waste water have been reported to produce asparaginase enzyme which is therapeutically and pharmaceutically useful. The isolates also showed antimicrobial, heamolytic activity and anticancer activity. The microbes producing asparaginase can also be optimized in natural substrates. The results of this study also revealed that utilization of coconut oil cake might be reduced the cost of therapeutically important L-asparaginase enzyme production.

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