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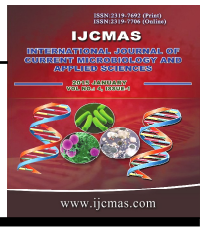

Original Research Article

Optimization of medium components for extracellular glutaminase free asparaginase from *Enterobacter cloacae*

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Keywords

Asparaginase, Glutaminase, Optimization, *E. cloacae*, Acute lymphoblastic leukemia

A B S T R A C T

Asparaginase is an important component for the treatment of acute lymphoblastic leukemia and other tumor malignancies, used more than four decade. The currently available asparaginases for chemotherapeutic applications possess intrinsic glutaminase activity due to which patients suffer with several severe side effects. In this investigation, physical and nutritional conditions were optimized for asparaginase production from *Enterobacter cloacae*. Maximal yield of asparaginase were recorded with batch time 21 h, inoculum age of 15 h, inoculum size 2%, initial pH 7.0 and temperature 40°C. Pyruvate, yeast extract, magnesium ions and asparagine were found to be the best carbon, nitrogen, mineral ion and inducer, respectively for asparaginase production. The intrinsic glutaminase activity was not reported with crude as well as purified asparaginase, which can be medically more important. These results suggested that *E. cloacae* can be used for large scale production of glutaminase free asparaginase but more detailed studies are required for strengthening of our current findings.

Introduction

Bacterial asparaginase (L-Asparaginase amidohydrolase, E.C. 3.5.1.1) is a selective and highly effective chemotherapeutic agent extensively used over the world in first-line treatment of acute lymphoblastic leukemia (ALL), Hodgkin disease, acute myelocytic leukemia, acute melanomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, reticlesarcoma and melanosarcoma (Verma *et al.*, 2007). The anti-neoplastic action of asparaginase is explained on the fact that certain tumor cells, more specifically ALL tumor cells are deficient in their ability to synthesize the non-essential amino acid asparagine *de-novo* due to absence of asparagine synthetase (Asselin *et al.*, 1989) but they require huge amount of asparagine to keep up their rapid malignant growth. To fulfill their nutritional requirement they use serum and cerebrospinal fluid (CSF) asparagine. The administration of asparaginase as a chemotherapeutic drug rapidly hydrolyses serum as well as CSF asparagine into aspartate and ammonia (Lubkowski *et al.*,

1996). The nutritional stress induced by asparaginase by depletion of serum as well CSF asparagine leads to DNA, RNA and protein biosynthesis inhibition in ALL and other asparagine dependent tumor cells, resulting in subsequent apoptosis due to cell cycle arrest in G1 phase (Gong *and* Basilico, 1990). However, normal cells are unaffected due to presence of asparagine synthetase (Narta *et al.,* 2007).

Since, it was demonstrated that asparaginase is a component of guinea pig serum and inhibited the proliferation of asparagine dependent certain tumors (Broome, 1961), various microorganisms such as *Bacillus licheniformis* (Mahajan *et al.,* 2014), *Bacillus aryabhattai* ITBHU02 (Singh *et al.,* 2013), *Penicillium digitatum* (Shrivastava *et al.,* 2012), *Erwinia carotovora* (Devi and Azmi, 2012), *Pectobacterium carotovorum* MTCC1428 (Kumar *et al.,* 2011), *Streptomyces thermoluteus* NBRC14270 (Hatanaka *et al.,* 2011), *Aspergillus niger* AK10 (Dharmsthiti and Luechai, 2010), *Serratia marcescens* SK-07 (Agarwal *et al.,* 2010), *Aspergillus terreus* (Mishra, 2007), *Pseudomonas aeruginosa* (Abdel-Fattah and Olama, 2002), *Escherichia coli* (Khushoo *et al.,* 2004) and *Enterobacter aerogenes* (Mukherjee *et al.,* 1999) were reported as asparaginase producer.

Unfortunately, till date asparaginase purified from *Escherichia coli* (EcAII) and *Erwinia chrysanthemi* (ErA) has been used for clinical purposes (Kozak and Jurga, 2002) due to their prolong serum half life, high affinity towards asparagine and low intrinsic glutaminase activity (3–10%).

The glutaminase activity of asparaginase is responsible for irreversible conversion of blood glutamine into glutamate and ammonia. This glutamate reacts with blood sodium and monosodium glutamate is formed (Kurtzberg *et al.,* 2003). Several recent reports suggested that due to glutaminase activity of asparaginase patients suffers with several life threatening side effects such as leucopenia, immunosuppression, acute pancreatitis, thromboembolysis, hyperglycemia and neurological seizures (Ramya *et al.,* 2011; Kravchenko *et al.,* 2008). Therefore, the search of glutaminase free asparaginase from indigenous microorganisms is still representing an actual scientific task.

Each bacterial species has its own particular nutritional and environmental requirement for maximum asparaginase synthesis. Therefore, no specific medium has been developed for the optimum production of asparaginase from different bacterial species. Screening and evaluation of the physical and nutritional requirements of microorganism are important stages to develop and determine overall economic feasibility of bioprocess (Chidambaram *et al.,* 2009).

The objective of this study is to optimize the various environmental and nutritional sources for maximize the production of asparaginase from *Enterobacter cloacae*.

Materials and Methods

Anhydrous L-asparagine, L-glutamine, sucrose, maltose, starch, galactose, lactose, melibiose, glucose, xylose, pyruvate, gelatin, tryptone, beef extract, ammonium oxalate, potassium nitrate, casein, ammonium chloride, urea, yeast extract, NaCl, $CaCl_2$, K_2HPO_4, $MgSO_4$, KCl, trichloroacetic acid (TCA), and Folin-Ciocalteu's phenol reagent were purchased from Himedia, Mumbai, India. All other chemicals used were of analytical grade and purchased from standard sources.

Bacterial strain and culture condition

The glutaminase free asparaginase producing strain *E. cloacae* (NCBI accession no: KF607094) was obtained from Bacterial Culture Collection Centre (BGCC no: 2389) from Rani Durgavati University, Jabalpur (M.P.), India, which was previously isolated by Sharma *et al.* (2014).

The strain was maintained on Luria-Bertani (LB) agar slant (pH 7) and stored at 4°C. Stock culture was transferred to fresh LB agar slant after every 4 weeks. The production of asparaginase was performed in modified M-9 medium containing (gL^{-1}) glucose, 3 g; $Na_2HPO_4 \cdot 2H_2O$, 6.0 g; KH_2PO_4, 3g; NaCl, 0.5g; $MgSO_4 \cdot 7H_2O$, 0.5g; $CaCl_2 \cdot 2H_2O$, 0.015; asparagine, 3 g with an initial pH of 7.0 (Gulati *et al.* 1997).

Optimization of asparaginase under shake flask culture

The asparaginase production from *E. cloacae* was optimized under shake flask culture according to the method of Kenari *et al.* (2011). The effect of different production parameters including batch time, inoculum age and size, pH, temperature, carbon, nitrogen, mineral ions and amino acids on enzyme production were studied. All experiments for optimization were performed in triplicates.

Primary inoculum preparation and batch time

For inoculum preparation, a loopfull of 24 h old pure culture of *E. cloacae* was transferred in 20 ml of aforementioned sterile medium and flask was incubated overnight at 37°C in a rotary shaking incubator (Remi C-24 BL) at 180 rpm. In order to determine batch time, 2% (v/v) inoculum (A600 = 0.6–0.8) of this culture was inoculated in 100 ml of modified M-9 broth medium and incubated at 37 ºC with shaking at 180 rpm. After 3 h of regular interval, 5 mL medium was withdrawn, centrifuged at 10,000 rpm and supernatant was subjected for asparaginase activity.

Effect of age and inoculum size

In order to determine the age and size (%) of inoculum on the level of asparaginase production, inoculum of different ages (5, 10, 15, 20, 25 and 30 h) and different sizes (1, 1.5, 2, 2.5, 3, 3.5 and 4%, v/v) was used to inoculate in 250 ml flask containing 30 mL minimal medium. Flasks were incubated at optimized incubation period at 37ºC with shaking at 180 rpm and asparaginase activity was analyzed.

Effect of pH and temperature

The effect of different pH (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9) and different temperatures (20, 25, 30, 35, 37, 40, 45 and 50°C) on asparaginase activity was investigated. The medium with pH 7.0 and temperature 37°C were set as a control. Asparaginase activity was analyzed by standard asparaginase assay.

Effect of carbon and nitrogen sources

To determine the influence of different carbon sources on asparaginase activity, various carbon sources (0.3%) (sucrose, maltose, starch, galactose, lactose, melibiose, xylose and pyruvate) were substituted in the medium in place of glucose. Then, to study the effect of different nitrogen sources on asparaginase activity various nitrogen sources (0.5%) (gelatin, tryptone, beef extract, ammonium oxalate, potassium nitrate, casein, ammonium chloride, urea and yeast extract) were substituted in the modified M-9 broth medium, individually. Asparaginase activity was analyzed by standard asparaginase assay.

Effect of mineral ions and amino acids

The effect of various ions sources such as NaCl, $CaCl_2$, K_2HPO_4, $MgSO_4$ and KCl (0.3) were used in the medium individually, and flasks were incubated at aforementioned optimized process parameters at 180 rpm. Asparaginase activity was analyzed by standard asparaginase assay. Then, to study the effect of different amino acids (L-form) (histidine, lysine, ornithine, asparagine, aspartic acid, glutamine, and arginine) on asparaginase activity, various amino acids (0.3%) were substitute individually in place of asparagine. Flasks were inoculated and incubated at aforementioned optimized conditions and asparaginase activity was analyzed.

Determination of asparaginase activity

The method of Wriston, (1970) was followed to determine the asparaginase activity of the isolates using Nesslerization reaction. This method utilizes the determination of ammonia librated from asparagine hydrolysis. The reaction mixture containing 0.1 mL crude enzyme preparation was added into 0.9 mL of pre-warmed 0.01M asparagine prepared in 0.05M Tris-HCl buffer (pH 8.6). The tube content was mixed by vortexing and incubated at 37°C for 30 min. The reaction was terminated by the addition of 0.1 mL of 1.5 M trichloroacetic acid (TCA).

The reaction mixture was centrifuged at 10,000 rpm for 5 min at room temperature to remove the precipitate. Nessler's reagent (0.25 mL) was added to the tubes containing 0.5 mL supernatant and 1.75 mL distilled water. The content of the tubes were vortexed and incubated at room temperature for 10 min. The absorbance A_{480} values were measured against the control prepared by addition of trichloroacetic acid (TCA) before enzyme addition. The ammonia produced in the reaction was calculated on the basis of standard curve prepared with ammonium sulphate. One international unit of asparaginase (IU) is defined as the amount of enzyme that liberates 1µmol of ammonia min^{-1} at 37°C. Specific activity of asparaginase is expressed as U mg^{-1} protein.

Glutaminase assay

Glutaminase assay was performed by following the standard method of Imada *et al.* (1973). A reaction mixture containing 0.5 mL of 0.5M Tris-HCl buffer (pH 7.5), 0.5 mL of crude enzyme preparation and 1 ml distilled water was incubated at 37°C for 30 min and reaction was stopped by adding 0.5 mL of 1.5 M trichloroacetic acid. Control was prepared by adding trichloroacetic acid prior to the addition of enzyme preparation.

The reaction mixture was centrifuged at 10,000 rpm for 5 min at room temperature to remove the precipitate. 0.1 mL of the above reaction mixture and 0.2 mL of Nessler's reagent was added in a tube containing 3.7 mL distilled water. After incubating the reaction mixture at 37°C for 30 min, optical density was measured at 450 nm (UV-Visible spectrophotometer, Systronic-2375).

Determination of protein content

The total protein content of the samples was determined according to the method of Lowry *et al.* (1951), using bovine serum albumin (BSA) as standard.

Results and Discussion

Effect of batch time

In order to evaluate the effect of batch time on asparaginase synthesis, culture flask was incubated at 37°C and 180 rpm. After 3 h, 5 mL culture was withdrawn and supernatant

was subjected for asparaginase activity. Results showed that 21 h of incubation was more suitable for asparaginase production (1.3±0.04 IU mL^{-1}) (Table 1). However, extended incubation period led to the low yield of asparaginase activity.

Effect of age and size of inoculum

As seen from Table 2 and 3, inoculum age of 15 h (1.43±0.06 IU mL^{-1}) and inoculum size of 2% (v/v) (2.46±0.10 IU mL^{-1}) were found to be the most suitable for maximum asparaginase yield. However, asparaginase yield was decreased at lower or higher inoculum age and inoculum size.

Effect of pH and temperature

The asparaginase production was studied over a pH range of 5.0-9.0. Maximum asparaginase yield 3.7±0.12 IU mL^{-1} was achieved at pH of 7.0 followed by pH of 7.5 which was 3.02±0.17 IU mL^{-1}. Indeed, further on increasing the pH, enzyme yield was decreased in pH dependent manner (Table 4). The result presented in Table 5 showed that maximum asparaginase production was achieved at 40°C which was 4.31±0.15 IU mL^{-1}. On further increasing temperature, enzyme activity was decreased in temperature dependent manner, which may be due to thermal denaturation of enzyme. However, lowest enzyme yield was found at 20°C which was 0.61±0.12 IU mL^{-1}.

Effect of carbon and nitrogen sources

The results showed that various carbon sources was able to support maximal asparaginase expression but pyruvate was responsible for maximum (7.01±0.15 IU mL^{-1}) enzyme yield followed by lactose (6.21±0.13 IU mL^{-1}) (Fig. 1). The effect of different nitrogen compounds on the production of asparaginase by this strain was studied by incorporating of different nitrogen sources into minimal medium, individually in place of glucose. Among them, culture medium emended with yeast extract (9.80±0.32 IU mL^{-1}) has affirmative effect on enzyme production followed by tryptone (6.93±0.27 IU mL^{-1}). The effect of different nitrogen sources is summarized in Figure 2.

Effect of mineral ions and amino acids

Different mineral ion sources were incorporated in minimal medium individually to determine their effect on asparaginase production. Results revealed that maximum enzyme yield 11.76±0.39 IU mL^{-1} was achieved with $MgSO_4$ **(**Fig. 3). The effect of various amino acids on asparaginase production by *E. cloacae* is summarized in Figure 4. The data revealed that maximal asparaginase yield 13.06±0.37 IU mL^{-1} was obtained by incorporation of asparagine.

Asparaginase is an integral part of combination chemotherapy protocols of pediatric ALL and other tumor malignancies, used world widely (Narta *et al.,* 2007) since last 40 years (Chan *et al.,* 2014). Today's rather than ALL therapy, asparaginase is also used in many other clinical experiment relating to tumor therapy due to which its demand increased tremendously and will be continue in forthcoming years (Geckil *et al.,* 2006). Indeed, low productivity and high bioprocessing cost of this drug is restricted its clinical applications. Biological production of any enzyme is a highly complex process, which involves several catalytic reactions and regulatory parameters at environmental, biochemical and genetic level (Prakasham *et al.,* 2006). Screening and evaluation of the environmental and

nutritional requirements of microorganisms are important steps for asparaginase production. In present investigation, various environmental and nutritional parameters were optimized for maximum synthesis of asparaginase from *E. cloacae*. This bacterium previously isolated from rhizospheric soil of *Calendula officinalis* and identified by Sharma *et al.* (2014).

The batch time profile showed that maximum enzyme yield was achieved during late stationary phase (21 h) while, trace enzyme activity was found at 9 h of incubation. This short fermentation time can be more significant for large scale production of enzyme. The inoculum age and inoculum percent directly influence the enzyme production. In this investigation, we observed that 1.1 and 1.8 fold increase in asparaginase yield, when 15 h old 2% inoculum was used. This is because that the *E. cloacae* achieved logarithmic growth after 15 h of incubation and the reduction of enzyme activity at inoculum size higher than 2% can be attributed to decrease in the concentration of the medium components (Kenari *et al.*, 2011).

The effect of pH on enzyme production was examined at various pH values ranging from 5-9. At pH 7.0 the yield of asparaginase was increased 2.8 fold. This might be due to that *E. cloacae* grow rapidly at near neutral pH or at this pH *E. cloacae* asparaginase might be more stable. However, below and above this pH enzyme yield was decreased abruptly. The adverse effect of acidic medium might be responsible for low production of asparaginase. These results are in accordance with the previous findings of Nawaz *et al.* (1998) and Geckil and Gencer (2004), they reported that the production of asparaginase from *Enterobacter cloacae* was down regulated linearly with pH of the medium, and the optimal activity was observed at neutral and alkaline pH.

In this study, the effect of different carbon sources on production of asparaginase was investigated. We observed that pyruvate was the best carbon source for asparaginase production which enhanced 5.3 fold yields. However, in the presence of glucose enzyme yield was just half as compared to pyruvate. This may be due catabolic repressor nature of this sugar or production of various acids by glucose catabolism which leads the lowering of medium pH. In the presence of glucose, low asparaginase yield were also reported with *Enterobacter aerogenes* (Mukherjee *et al.,* 2000), *Erwinia carotovora* (Warangkar and Khobragade, 2009) and *Escherichia coli* (Kenari *et al.*, 2011). While, from *Klebsiella aerogenes* glucose does not inhibit the production of this enzyme (Resnik and Magasanik, 1976).

Asparaginase production from this strain varied with various compound tested as nitrogen sources. Among them, culture medium supplemented with yeast extract (7.5 fold) favored maximum asparaginase synthesis followed by tryptone (5.3 fold). In bacterium *Erwinia aroideae* (Liu and Zajic, 1973), yeast extract supported high yield of asparaginase while, tryptone and yeast extract stimulated asparaginase synthesis in *Erwinia carotovora* and *Escherichia coli* (Maladkar *et al.,* 1993; Kenari *et al.*, 2011).

To select the most favorable mineral ion source for the enhanced production of asparaginase, experiments were performed using various mineral ion sources. The presence of Mg^{2+} (magnesium sulphate) ions in the medium improved the enzyme productivity approximately 9 fold. The presence of asparagine in the medium improved the enzyme productivity approximately 10 fold. However, rest of all the inducers, glutamine and arginine were also found to improve asparaginase synthesis 8.4 and 6.9 fold, respectively.

These results indicated that asparaginase produced from *E. cloacae* was an inducible enzyme. These results are in the accordance with previous report of Singh and Srivastava, (2013), who reported 3 fold, increased of extracellular asparaginase synthesis from *Bacillus aryabhattai* ITBHU02 when medium supplemented with asparagine.

Asparaginase is an important anticancer agent world widely used in first and second line treatment of acute lymphoblastic leukemia (Pieters *et al.,* 2007) and lymphoproliferative malignancies (Narta *et al.,* 2007). Currently, *E. coli* and *E. chrysanthemi* asparaginases are used in clinical practices. Unfortunately, asparaginase obtained from both sources showed intrinsic glutaminase activity due to which patients suffer with several life threatening side effects (Masetti and Pession, 2009). The intrinsic glutaminase activity with crude as well as purified (the data of purification is not shown) *E. cloacae* asparaginase did not detected. Therefore, this unique property suggested that purified enzyme is different from other known asparaginases that previously reported from various bacterial species. Therefore, *E. cloacae* asparaginase could be used as an ideal antileukemic agent for the treatment of acute lymphoblastic leukemia and other lymphoproliferative disorders.

In conclusion, present study demonstrated that optimization of cultural and nutritional conditions for production of asparaginase from *E. cloacae*. The biosynthesis of asparaginase from this strain was maximum when batch time 21 h, inoculum age of 15 h, inoculum size 2%, initial pH 7.0 and temperature 40°C was used. However, pyruvate, yeast extract, magnesium ions and asparagine were best carbon, nitrogen, mineral ions and inducer sources, respectively. *E. cloacae* asparaginase did not exhibit intrinsic glutaminase activity. The results of present study suggested that *E. cloacae* could be used for large scale production of glutaminase free asparaginase.

Table.1 Effect of batch time on asparaginase production from *E. cloacae*. Strains were inoculated in 100 mL medium and incubated on rotary shaking incubator at 37°C and 180 rpm

Batch time (h)	Asparaginase activity (IU mL^{-1})
9	0.2±0.04
12	0.3±0.05
15	0.69±0.07
18	1.0±0.05
21	1.3±0.04
24	1.2±0.05
28	1.1±0.03

Values are the means of three replicates ± SD.

Table.2 Effect of inoculum ages on asparaginase production from *E. cloacae*. Different ages of inoculum were inoculated in 50 mL medium and incubated on rotary shaking incubator at 37°C and 180 rpm

Inoculum age (h)	Asparaginase activity (IU mL^{-1})
5	0.62±0.05
10	0.95±0.08
15	1.43±0.06
20	1.32±0.05
25	1.02±0.08
30	0.71±0.06

Values are the means of three replicates ± SD.

Table.3 Effect of inoculum size on asparaginase production from *E. cloacae*. Different size (%) of inoculum was inoculated in 50 mL medium and incubated on rotary shaking incubator at 37°C and 180 rpm

Inoculum size (%)	Asparaginase activity (IU mL^{-1})
1	0.54±0.05
1.5	1.76±0.07
2	2.46±0.1
2.5	2.23±0.09
3	1.86±0.12
3.5	1.23±0.07
4	0.68±0.07

Values are the means of three replicates ± SD.

Table.4 Effect of pH on asparaginase production from *E. cloacae*. 2% inoculum was inoculated in 50 mL medium of different pH and flasks were incubated on rotary shaking incubator at 37°C and 180 rpm

pH	Asparaginase activity (IU mL^{-1})
5	0.32±0.1
5.5	0.53±0.13
6	1.68±0.08
6.5	2.38±0.16
7	3.70±0.12
7.5	3.02±0.17
8	2.45±0.12
8.5	1.59±0.16
9	0.98±0.12

Values are the means of three replicates ± SD.

Table.5 Effect of temperature on asparaginase production from *E. cloacae*. 2% inoculum was inoculated in 50 mL medium (pH 7.0) and flasks were incubated on rotary shaking incubator at different temperature at 180 rpm

Temperature (°C)	Asparaginase activity (IU mL^{-1})
20	0.61±0.12
25	0.82±0.14
30	2.31±0.17
35	3.31±0.13
37	4.01±0.15
40	4.31±0.15
45	2.52±0.17
50	0.89±0.13

Values are the means of three replicates ± SD.

Fig.1 Effect of carbon sources on asparaginase production from *E. cloacae*. Strains were incubated in 50 mL of medium in 250 mL Erlenmeyer flask on rotary shaking incubator at 180 rpm for 21 h

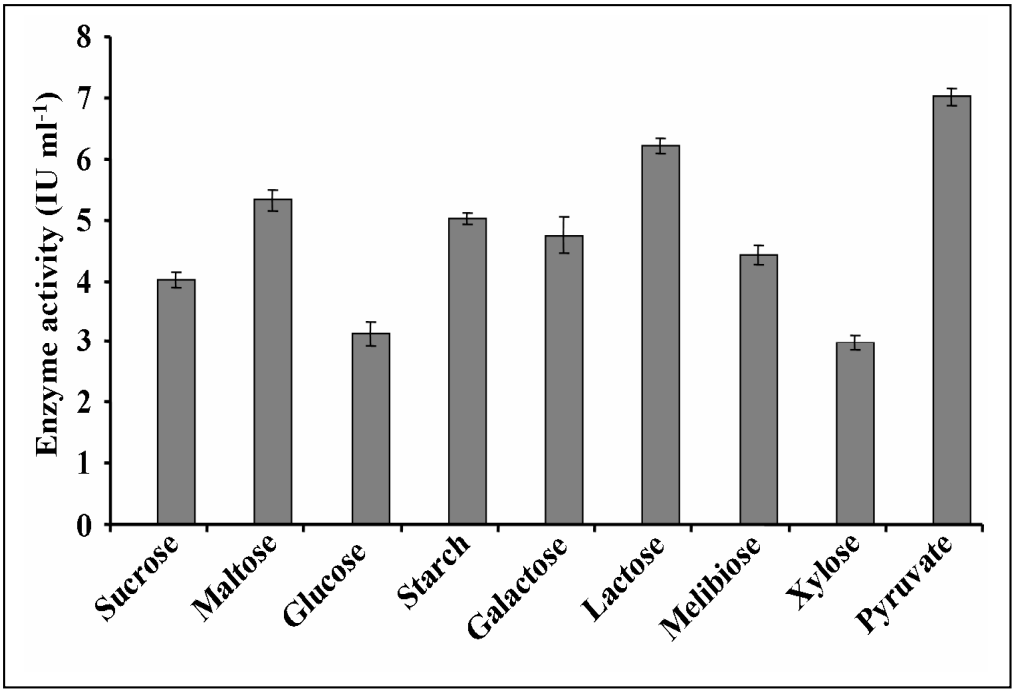


Fig.2 Effect of various nitrogen sources on asparaginase production from *E. cloacae*. Strains were incubated in 50 mL of medium in 250 mL Erlenmeyer flask on rotary shaking incubator at 180 rpm for 21 h

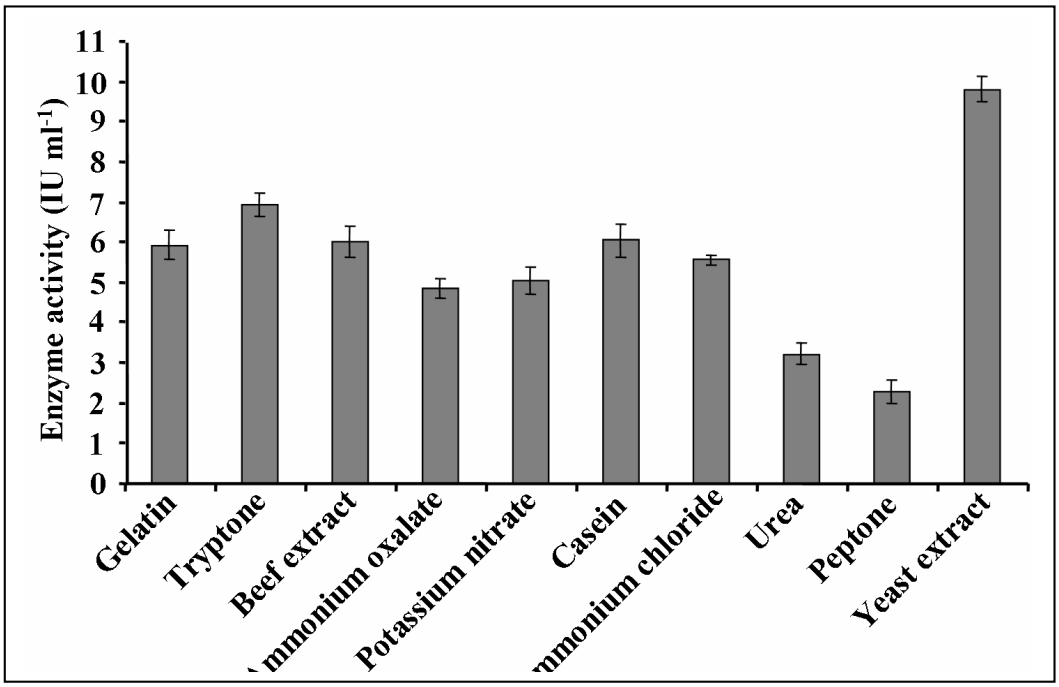


Fig.3 Effect of various mineral ions on asparaginase production from *E. cloacae*. Strains were incubated in 50 mL of medium in 250 mL Erlenmeyer flask on rotary shaking incubator at 180 rpm for 21 h

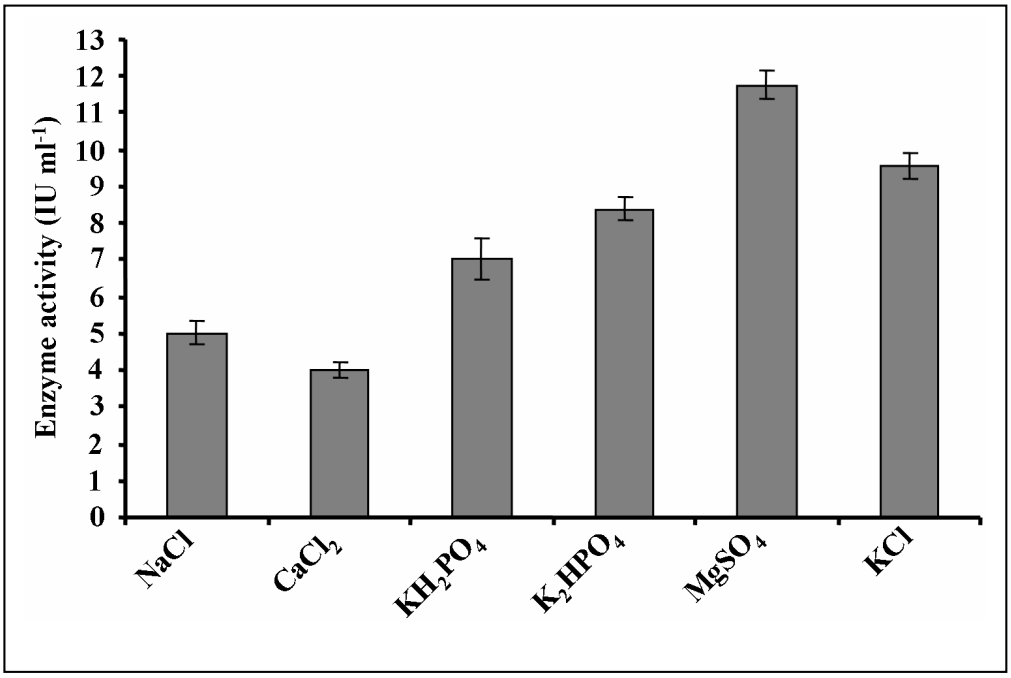
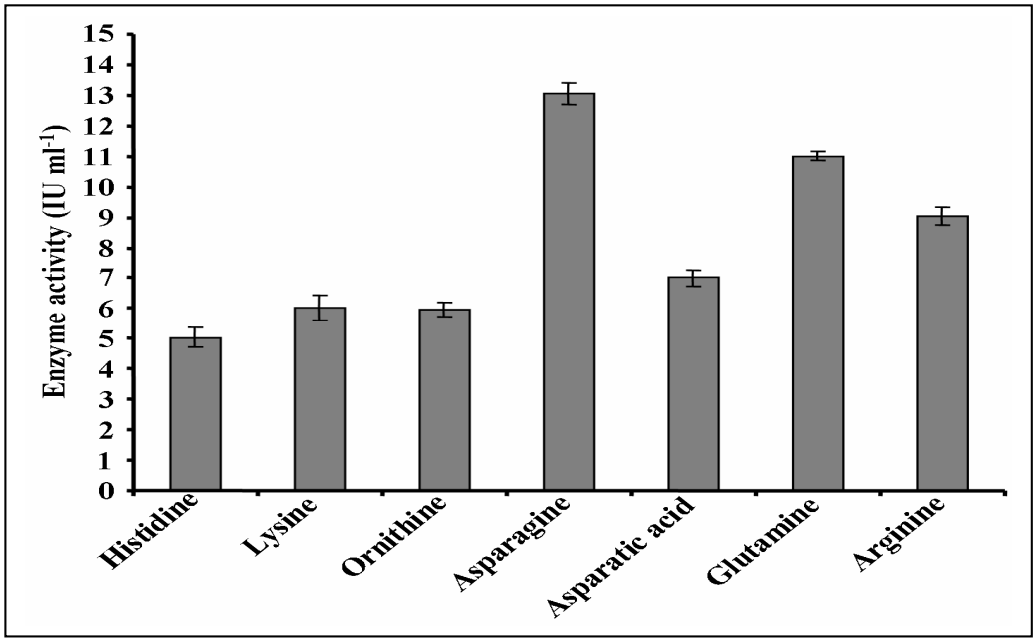


Fig.4 Effect of amino acids on asparaginase production from *E. cloacae*. Strains were incubated in 50 mL of medium in 250 mL Erlenmeyer flask on rotary shaking incubator at 180 rpm for 21 h



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References

Abdel-Fattah, Y.R., Olama, Z.A. 2002. L-Asparaginase production by *Pseudomonas aeruginosa* in solid-state culture: evaluation and optimization of culture conditions using factorial designs. *Process Biochem.,* 38: 115– 122.

Agarwal, A., Kumar, S., Veeranki, V.K. 2010. Effect of chemical and physical parameters on the production of L- asparaginase from a newly isolated *Serratia marcescens* SK- 07. *Lett. Appl. Microbiol.,* 52: 307–313.

Asselin, B.L., Ryan, D., Frantz, C.N., Bernal, S.D., Leavitt, P., Golbey, R.D., Krakoff, I.H., Karnofsky, D.A., Burchen– Sallan, S.E., Cohen, H.J. 1989. *In vitro* and *in vivo* killing of acute lymphoblastic leukemia cells by L- asparaginase. *Cancer Res.* 49: 4363– 4368.

Broome, J.D. 1961. Evidence that the L-asparaginase activity in guinea pig serum is responsible for its antilymphoma effects. *Nature,* 19: 14–25.

Chan, W.K., Lorenzi, P.L., Anishkin, A., Purwaha, P., Rogers, D.M., Sukharew, S., Rempe, S.B., Weinstein, J.N. 2014. The glutaminase activity of L-asparaginase is not required for anticancer activity against ASNS–negative cells. *Blood,* 21: 1–26.

Chidambaram, K.V., Kuppanan, N., Kannan, K., Perumalsamy, L. 2009. Production of L- asparaginase by *Serratia marcescens* SB08: optimization by response surface methodology. *Iran J. Biotechnol.,* 7: 10–30.

Devi, S., Azmi, W. 2012. One step purification of glutaminase free asparaginase from *Erwinia carotovora* with anticancrous activity. *Pharm. Sci.*, 2: 36– 45.

Dharmsthiti, S.C., Luechai, S. 2010. Purification and characterization of asparaginase from solid state culture of *Aspergillus niger* AK 10. *Int. J. Biotechnol. Biochem.,* 6: 1083–1092.

Geckil, H., Gencer, S. 2004. Production of L- asparaginase in *Enterobacter aerogenes* expressing *Vitreoscilla* hemoglobin for efficient oxygen uptake. *Appl. Microbiol. Biotechnol.,* 63: 691– 697.

Geckil, H., Gencer, S., Ates, B., Ozer, U., Uckun, M., Yilmaz, I. 2006. Effect of Vitreoscilla hemoglobin on production of a chemotherapeutic enzyme, L- asparaginase, by *Pseudomonas aeruginosa*. *Biotechnol. J.*, 1: 203–208.

Gong, S.S., Basilico, C. 1990. A mammalian temperature– sensitive mutation affecting G1 progression results from a single amino acid substitution in asparagine synthetase. *Nucleic Acids Res.,* 18: 3509–3513.

Gulati, R., Saxena, R.K., Gupta, R.A. 1997. Rapid plate assay for screening L- asparaginase producing micro-organisms. *Lett. Appl. Microbiol.*, 24: 23–26.

Hatanaka, T., Usuki, H., Arima, J., Uesugi, Y., Yamamoto, Y., Kumagai, Y., Yamasato, A., Mukaihara, T. 2011. Extracellular production and characterization of two *Streptomyces* L- asparaginases. *Appl. Biochem. Biotechnol.,* 163: 836–844.

Imada, A., Igarasi, S., Nakahama, K., Isono, M. 1973. Asparaginase and Glutaminase activities of microorganisms. *J. Gen. Microbiol.*, 76, 85–99.

Kenari, S.L.D., Alemzadeh, I., Maghsodi, V. 2011. Production of L- asparaginase from *Escherichia coli* ATCC 11303: optimization by response surface methodology. *Food Bioprod. Proc.*, 89: 315–321.

Khushoo, A., Pal, Y., Singh, B.N., Mukherjee, K.J. 2004. Extracellular expression and single step purification of recombinant *Escherichia coli* L- Asparaginase II. *Prot. Expr. Purif.,* 38: 29–36.

Kozak, M., Jurga, S. 2002. A comparison between the crystal and solution structures of Escherichia coli asparaginase II. *Acta Biochim. Pol.,* 49: 509–513.

Kravchenko, O.V., Kislitsin, Y.A., Popov, A.N., Nikonov, S.V., Kuranova, I.P., 2008. Three dimensional structures of L- asparaginase from *Erwinia carotovora* complexed with aspartate and glutamate. *Acta Crystallog. D*, 64: 248–256.

Kumar, S., Dasu, V.V., Pakshirajan, P. 2011. Purification and characterization of glutaminase–free L- asparaginase from *Pectobacterium carotovorum* MTCC 1428. *Bioresour. Technol.,* 102: 2077–2082.

Kurtzberg, J., Yousem, D., Beauchamp, N., 2003. Asparaginase, Chapt. 55. In: Kufe, D.W., Pollock, R.E., Weichselbaum, R.R., et al., (Eds.), Holland-Frei Cancer Medicine, 6th edn. BC Decker, Hamilton (ON), Pp.

699–705.

Liu, F.S., Zajic, J.E. 1973. Fermentation kinetics and continuous process of L- asparaginase production. *Appl. Microbiol.,* 25(1): 92–96.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* 193: 265–275.

Lubkowski, J., Palm, G.J., Gilliland, G.L., Derst, C., Rohm, K.H. 1996. Crystal structure and amino acid sequence of *Wolinella succinogenes* L-asparaginase. *Eur. J. Biochem.,* 241: 201–207.

Mahajan, R.V., Kumar, V., Rajendran, V., Saran, S., Ghosh, P.C., Saxena, R.K. 2014. Purification and characterization of a novel and robust L- asparaginase having low–glutaminase activity from *Bacillus licheniformis*: *in vitro* evaluation of anti–cancerous properties. *PLoS ONE,* 9: 1–8.

Maladkar, N.K., Singh, V.K., Naik, S.R. 1993. Fermentative production and isolation of L- asparaginase from *Erwinia cartovora* EC–113. *Hidustan Antibiot. Bull.,* 35: 77–86.

Masetti, R., Pession, A. 2009. First–line treatment of acute lymphoblastic leukemia with pegasparaginase. *Biologics,* 3: 359–368.

Mishra, A. 2007. Production of L-asparaginase, an anticancer agent, from *Aspergillus niger* using agricultural waste in solid state fermentation. *Appl. Biochem. Biotech.,* 135: 33–42.

Mukherjee, J., Joeris, K., Riechel, P.,Scheper, T. 1999. A simple method for the isolation and purification of L- asparaginase from *Enterobacter aerogenes*. *Folia Microbiol (Praha).* 44: 15–28.

Mukherjee, J., Majumadar, S., Scheper, T., 2000. Studies on nutritional and oxygen requirements for production of L- asparaginase by *Enterobacter aerogenes. Appl. Microbiol. Biotechnnol.,* 53: 180–184.

Narta. U.K., Kanwar, S.S., Azmi, W. 2007. Pharmacological and clinical evaluation of L- asparaginase in the treatment of leukemia. *Crit. Rev. Oncol. Hematol.,* 61: 208–221.

Nawaz, M.S., Zhang, D., Khan, A.A., Cerniglia, C.E. 1998. Isolation and characterization of Enterobacter cloacae capable of metabolizing asparagine. *Appl. Microbiol. Biotechnol.,* 50: 568–572

Pieters, R., Hunger, S.P., Boos, J., Rizzari, J., Silverman, L., Baruchel, A., Goekbuget, S.N., Pui, C. 2007. L-Asparaginase treatment in acute lymphoblastic leukemia. *Cancer,* 26: 238–249.

Prakasham, R.S., Rao, Ch, C.S., Sreenivas, Rao, R., Sharma, P.N. 2006. Enhancement of acid amylase production by an isolated *Aspergillus awamori*. *J. Appl. Microbiol.,* 92: 1365–2672.

Ramya, L.N., Doble, M., Rekha, V.P.B., Pulicherla, K.K. 2011. L-Asparaginase as potent antitumor agent and its significance of having reduced glutaminase side activity for batter treatment of acute lymphoblastic leukemia. *Appl. Biotech. Biotechnol.,* 167: 2144–2159.

Resnik, A., Magasanik, B. 1976. L-asparaginase of *Klebsiella aerogenes*. Activation of its synthesis by glutamine synthetase. *Biol. Chem.,* 251: 2722–2728.

Sharma, A., Husain, I., Mishra, S. 2014. Evaluation of antitumor activity of glutaminase–free L- asparaginase

from indigenous bacterial strains for potential chemotherapeutic application. *Int. J. Pharm. Bio. Sci. B,* 5: 16–26.

Shrivastava, A., Khan, A.A., Shrivastav, A., Jain, S.K., Singhal, P.K. 2012. Kinetic studies of L- asparaginase from *Penicillium digitatum*. *Prep. Biochem. Biotechnol.,* 42: 574–581.

Singh, Y., Gundampati, K.R., Jagannadham, M.V., Srivastava, S.K. 2013. Extracellular L-asparaginase from protease–deficient *Bacillus aryabhattai* ITBHU02: Purification characterization, and evaluation of antineoplastic activity *in vitro*. *Appl. Biochem. Biotechnol.,* 171: 1759–1774.

Verma, N., Kumar, K., Kaur, G., Anand, S. 2007. L- Asparaginase: A promising chemotherapeutic agent. *Crit. Rev. Biotechnol.,* 27: 45–62.

Warangkar, S.C., Khobragade, C.N. 2010. Purification, characterization, and effect of thiol compounds on activity of the *Erwinia carotovora* L-Asparaginase. *Enzyme Res.,* 11: 1–10.

Wriston, Jr. J.C. 1970. Asparaginase. *Methods enzymol.,* XVII: 732–742.