EXTRACELLULAR L-GLUTAMINASE PRODUCTION BY MARINE BREVUNDIMONAS DIMINUTA MTCC 8486

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Abstract

Maximal L-glutaminase enzyme production by *Brevundimonas diminuta* MTCC 8486 (48.4 U/ml) occurred at pH 6.0, 30°C in a sea water based medium supplemented with L-glutamine (1%, w/v), D-glucose (1.5%, w/v), peptone (1%, w/v), and potassium dihydrogen phosphate (1%, w/v) after 28 h of incubation and molecular weight of purified enzyme was 140 kDa.

Keywords : L-Glutaminase, *Brevundimonas diminuta*, marine bacteria

I. INTRODUCTION

L-Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2.) is the enzyme deamidating L-glutamine to L-glutamic acid and ammonia. Glutaminase is ubiquitous in microorganisms and it plays a major role in the cellular metabolism of both prokaryotes and eukaryotes. In general, glutaminases from *Escherichia coli*, *Pseudomonas* spp., *Rhizobium etli*, *Micrococcus luteus* K-3, *Bacillus* spp., *Clostridium welchii*, *Vibrio costicola*, *Zygosaccharomyces rouxii* and *Aspergillus oryzae* have been isolated and well studied. In recent years glutaminase has attracted much attention with respect to proposed applications in food industries and pharmaceutical industries (Yokotsuka, 1985; Sabu, 2003). The activity of glutaminase, which is responsible for the synthesis of glutamic acid, makes it an important additive during soy sauce fermentation. Attempts to increase the glutamate content of soysuace using a salt tolerant and thermo tolerant glutaminases have drawn much attention (Nandakumar *et al*., 2003). Commercial importance demands not only the search for new and better yielding microbial strains, but also economically viable bioprocesses for its large scale production. *Brevundimonas diminuta* is a non-lactose-fermenting environmental Gram-negative bacilli previously assigned to the genus *Pseudomonas*. Previous reports state that *B. diminuta* TPU 5720 produces an amidase acting L-stereoselectively on phenylalaninamide (Komeda *et al*., 2006). It has been identified that approximately 60 microbial species could produce L-glutaminase (Nandakumar *et al*., 2003). Until now there are no reports available for the presence of L-glutaminase from *B. diminuta*. Since the enzymes from marine microorganisms play an important role in both pharmaceutical and food industries, the present study aimed to produce L-glutaminase from marine bacterium, *B. diminuta* MTCC 8486. The study also included process optimization for production of L-glutaminase, enzyme purification and molecular weight determination.

II. MATERIALS AND METHODS

A. Isolation and identification of B. diminuta

The bacterium used in the present study B. diminuta was isolated from the sea water collected from the coastal area of Arabian sea, Trivandrum, Kerala, India. The culture was maintained at ZoBell's agar slants (peptone 5 g, yeast extract 1 g, FePO4 4H2O 0.01 g, agar 15 g, aged seawater 750 ml, distilled water 250 ml, pH 7.2) and subcultured every month (Park et al., 2002). B. diminuta strain was identified at Microbial Type Culture Collection (MTCC), Chandigarh, India and it was deposited in MTCC and assigned as *B. diminuta* MTCC 8486.

B. Inoculum Preparation

Inoculum prepared by growing the cells in marine broth (100 ml, composition as in Zobell's agar, but without agar) for 24 h at 30°C. The cells were pelleted by centrifugation at 15,000 rpm for 10 min. Pelleted cells were washed twice and resuspended in sterile saline and was used to inoculate the marine broth (Prabhu and Chandrasekaran, 1997).

C. Effect of process parameters on production of L-glutaminase

Marine broth (100 ml) was taken as a basal medium in 250 ml Erlenmeyer flask (shaken at 100 rpm) and the process parameters under study were varied. After optimization of each parameter, it was included in the next step at its optimal level. The parameters optimized were incubation time, initial pH of the medium (5–10), incubation temperature (20 – 45°C additional sodium chloride concentration (0–4%), additional carbon sources (glucose, fructose, sucrose, maltose, mannitol and sorbitol at 1%, w/v), additional nitrogen sources (peptone, yeast extract, beef extract, malt extract) additional inorganic salts (ammonium sulphate, ammonium nitrate, calcium nitrate, potassium dihydrogen phosphate at 1%, w/v) and different amino acids (L-glutamine, L-glutamic acid, L-asparagine, arginine, methionine, proline and lysine at 1%, w/v). Glutaminase was assayed according to Imada *et al.* (1973). Enzyme and substrate blanks were used as controls. One unit of L-glutaminase activity was defined as the amount of enzyme that liberated 1 μmol of ammonia under optimal assay conditions.

D. Purification and molecular weight determination of L-glutaminase

Enzyme isolation and purification was conducted at 0 – 7°C. The pH of the enzyme–containing cell extract was lowered to 6.5 by dropwise addition of phosphoric acid. Approximately 100 ml of supernatant fluid which contained about 4.47 g of protein and 560 U of L-glutaminase activity were applied to CM-Cellulose column (PHARMACIA FINE CHEMICALS) (5×80 cm) which had been equilibrated with 0.04 M sodium phosphate buffer, pH 6.5. The enzyme was eluted with 3 L liner gradient of 0 to 1 M NaCl in 0.04 M sodium phosphate buffer, pH 6.5, at a flow rate of about 10 ml per hour. Fractions of 1 ml were collected and assayed for L-glutaminase activity. Glutaminase was eluted from the CM-cellulose column at between 0.15 to 0.25 M NaCl concentration in a relatively small volume. The active fractions were combined and the pH of the pool was adjusted to 7.2 with sodium hydroxide. Solid ammonium sulfate (60%) was added slowly to the enzyme solution while maintaining pH 7.2 by dropwise addition of ammonium hydroxide. After 30 min at 4°C the precipitate was removed by centrifugation and suspended in 0.01 M sodium phosphate buffer, pH 7.2. The precipitate was dialyzed against the suspending buffer. The dialyzed enzyme solution (12.6 ml) which contained about 7.3 mg of protein and 160.02 U of L-glutaminase activity was adjusted to pH 8.0 with dilute NaOH and applied to a Sephadex column (G-200) (PHARMACIA FINE CHEMICALS) which has been equilibrated with 0.01 M sodium phosphate buffer, pH 8.0. The column was eluted with 0.01 M sodium phosphate buffer, pH 8.0, at a flow rate of 0.5 ml/minute (Roberts *et al.*, 1972). The glutaminase pool of fractions which appeared at the front was adjusted to pH 7.2 with dilute HCl. Protein concentration was analyzed by the method of Lowry *et al.* (1951). Bovine serum (1 mg/ml) was used as the standard. Molecular weight of purified L-glutaminase was determined by SDS-polyacrylamide gel electrophoresis with appropriate protein markers (Laemmli, 1971).

III. RESULTS AND DISCUSSION

Extracellular L-glutaminase production by *B. diminuta MTCC 8486* grown in shake flasks was observed in the present study. *B. diminuta* produced less L-glutaminase enzyme (18 U/ml) in nutrient broth compared to marine broth (22 U/ml) under same conditions after 24 h incubation period at 30°C (Data not shown). Hence, marine broth was selected as a basal medium to optimizing the process parameters for the production of L-glutaminase. From the Fig.1 it is clear that *B. diminuta* produces maximum L-glutaminase at 28 h of incubation. Data presented in the Fig. 2 clearly indicated the influence of initial pH of the medium on L-glutaminase production by *B. diminuta*. The optimum pH was observed at pH 6.0 (26.8 U/ml). Enzyme production increased along with increase in pH from 12.4 U/ml at pH 4.0 to a maximum of 26.8 U/ml at pH 6.0. Any further increase in the initial pH resulted in the reduction of enzyme production. Most of the extracellular enzymes are produced at higher levels at a growth pH that is near to the optimal pH required for the maximal enzyme activity (Tigue *et al.*, 1994).

Table 1. Effect of additives on L-glutaminase production by *B. diminuta* MTCC 8486

Additives (1%, w/v)	L-Glutaminase activity (U ml^{-1})
Carbon sources	
Control	33.5 ± 1.0
Glucose	35.1 ± 0.3
Fructose	34.0 ± 0.3
Sucrose	33.8 ± 0.2
Sorbitol	34.1 ± 0.1
Mannitol	34.3 ± 0.5
Maltose	34.4 ± 0.4
Organic nitrogen sources	
Control	38.2 ± 0.3
Peptone	42.6 ± 0.4
Yeas extract	36.2 ± 0.6
Beef extract	38.6 ± 0.2
Malt extract	38.1 ± 0.4
Inorganic salt sources	
Control	42.6 ± 0.4
Ammonium sulphate	40.2 ± 0.4
Sodium nitrate	41.2 ± 0.5
Calcium nitrate	39.4 ± 0.8
Potassium dihydrogen phosphate	44.3 ± 0.5
Ammonia acids	
Control	44.3 ± 0.5
L-Glutamine	48.4 ± 0.4
Glutamic acid	42.8 ± 0.6
Asparagine	37.2 ± 0.5
Arginine	26.0 ± 0.8
Methionine	28.0 ± 0.6
Proline	15.6 ± 0.4
Lysine	18.2 ± 0.6

Values are mean ± standard deviation; $n = 3$ samples. Optimized parameters are included in next step.

Incubation at 30°C, at pH 6.0 (optimized), slightly enhanced the enzyme production (27.2 U/ml) compared to other temperatures (Fig. 3). No growth of *B. diminuta* was observed at 40 and 45°C suggested that the bacterium is a mesophile (data not shown). Nevertheless, a considerable level of enzyme production could be obtained at other pH and temperatures. Prabhu and Chandrasekaran (1997) obtained maximal L-glutaminase yield by marine *Vibrio costicola* in solid state fermentation at 35°C and pH 7.0, after 24 h. Maximal extracellular L-glutaminase titres by *Zygosaccharomyces rouxii* were produced when solid state fermentation was carried out at 30°C incubation temperature and 48 h of incubation period (Kashyap *et al*., 2002). These factors are largely characteristic of the organism and vary for each species (Chandrasekaran *et al.,* 1991).

The salt dependence of glutaminase was determined by adding 0-5% (w/v) NaCl to the production medium. Glutaminase activity was increased from 27.2 to 33.5 U/ml at 2.5% NaCl. Addition of NaCl above 2.5% led to a decline in the enzyme production. (Fig. 4). Furthermore, in presence of 4% salt the enzyme activity retains still 87.5% compared to the reaction with 2.5% NaCl. No growth of *B. diminuta* was observed in the medium with above 4% NaCl (Data not shown). This indicates that the bacterium is not halophilic, but could be halotolerant and a natural commensal organism of the marine environment.

Results on the effect of supplementation of production medium with different carbon sources such as glucose, sucrose, maltose, sorbitol, fructose and mannitol on enzyme production are shown in the Table 1. Incorporation of additional carbon sources enhanced enzyme yield from 33.5 U/ml to 35.1 U/ml. Among the carbon sources tested D-glucose (1%) promoted maximal yield (35.1 U/ml) compared to others. Interestingly, supplementation of all the carbon sources

Table 2. Purification of *B. diminuta* MTCC 8486 L-glutaminase

Fraction	Volume (ml)	Total Protein (mg ml^{-1})	Total activity (U)	Specific activity (U mg^{-1})	Purification factor
Cell suspension	100	447	560	1.25	1
CM-cellulose	16	93.76	163.2	1.74	1.39
60% $(NH_4)_2SO_4$	12.6	7.3	160.02	21.92	17.53
Sephadex-G-200	1.4	0.36	156.4	60.15	48.12

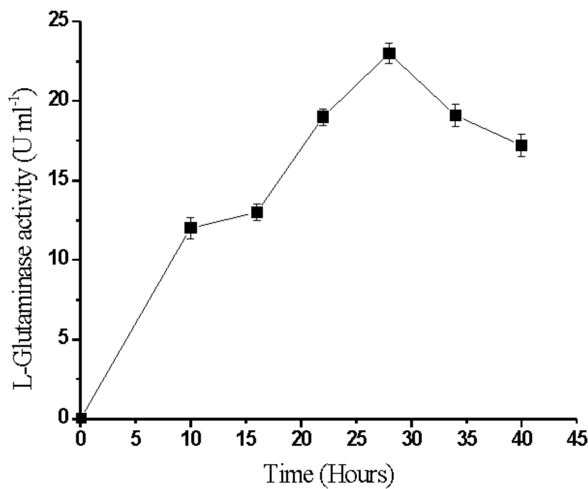

Fig. 1. Effect of incubation time on L-glutaminase production by *B. diminuta* MTCC 8486
Values are mean ± standard deviation;

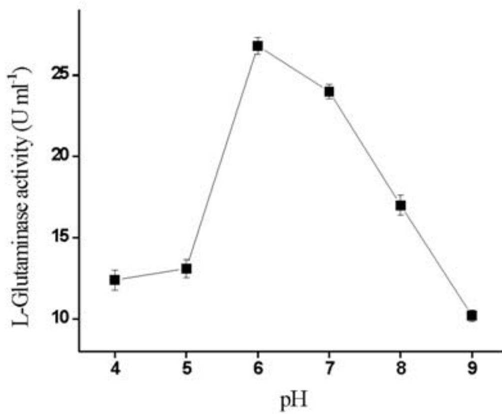

Fig. 2. Effect of initial medium pH on L-glutaminase production by ***B. diminuta* MTCC 8486**

Values are mean ± standard deviation;
n = 3 samples.

to the production medium enhances enzyme production by the marine bacteria. Sabu *et al.* (2000) reported the results on optimization of process parameters for the production of L-glutaminase by the marine fungus, *Beauveria bassiana* under solid state fermentation on an inert substrate. They found that among various carbon sources tested D-glucose at 0.5%, w/v almost doubled the glutaminase yield compared with others. Maltose (1%, w/v) was found to enhance L-glutaminase production by *V. costicola* under solid state fermentation (Prabhu and Chandrasekaran, 1997). The enhanced

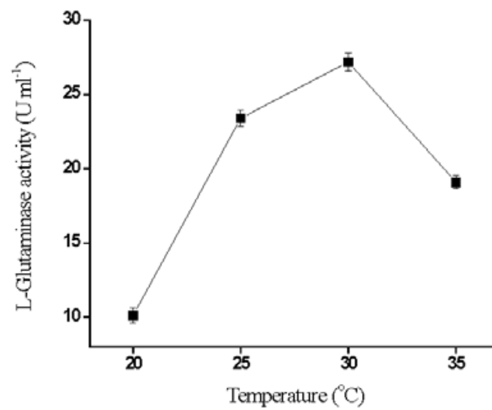

Fig. 3. Effect of incubation temperature on L-glutaminase production by *B. diminuta* MTCC 8486
Values are mean ± standard deviation;
n = 3 samples.

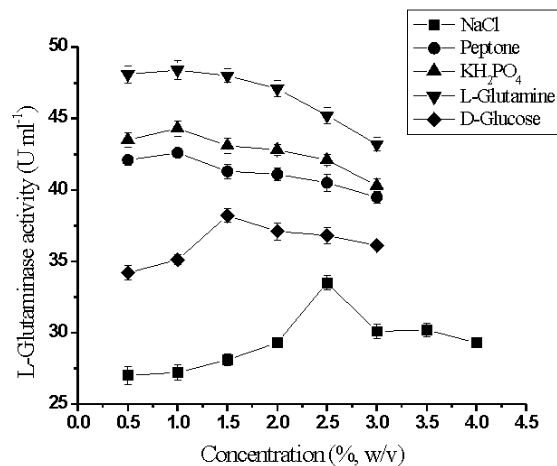

Fig. 4. Effect of additional NaCl, lactose, peptone, potassium dihydrogen phosphate and glutamine concentration on on L-glutaminase production by B. . diminuta MTCC 8486 diminuta MTCC 8486
Values are mean ± standard deviation;
n = 3 samples.

production of L-glutaminase by incorporation of carbon sources may be attributed to the positive influence of additional carbon sources along with glutamine on enhanced biosynthesis. Further studies were carried out for optimizing the concentration of glucose, which showed that 1.5% (w/v) glucose was optimal for maximum glutaminase (38.2 U/ml) (Fig. 4).

The results on the effect of addition of organic nitrogen sources, namely peptone, yeast extract, beef extract and malt extract on enzyme production after

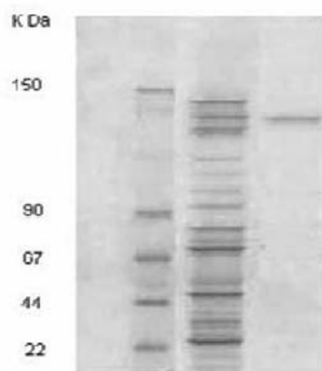

Fig. 5. SDS-PAGE of purified glutaminase from *B. diminuta* MTCC 8486.

Lanes: 1-Molecular markers, 2-crude enzyme, 3-purified glutaminase

28 h when they were incorporated in the medium at 1%, w/v level revealed that peptone enhanced the enzyme yield from 38.2 to 42.6 U/ml (Table 1). Further studies on peptone showed that 1% is the optimal concentration for the production of L-glutaminase by *B. diminuta* (Fig. 4). Nitrogen can be an important limited factor in the microbial production of enzymes (Chandrasekaran *et al*., 1991). It has also been reported that addition of yeast extract or tryptone to the growth medium resulted in significantly lower levels of enzyme activity (Roberts *et al.,* 1972).

Among the inorganic salt sources tested, only potassium dihydrogen phosphate was found to enhance the L-glutaminase production (44.3 U/ml). Ammonium sulphate, sodium nitrate and calcium nitrate were found to decrease the enzyme production at 1%, w/v concentration (Table 1). Further studies on potassium dihydrogen phophate concentration revealed that 1%, w/v is the optimal level for maximum enzyme yield (Fig. 4). This result emphasise the critical role of phosphate in the enhanced secretion of glutaminase. Among the different amino acids tested, L-glutamine was observed to enhance L-glutaminase synthesis (48.4 U/ml) (Table 1). This observation suggests that L-glutamine act as an inducer for the production of extracellular L-glutaminase enzyme. L-Glutaminase production occurred even in the absence of L-glutamine as well as any additional amino acid in the seawater medium. This particular observation suggests that *Brevundimonas diminuta* could produce extracellular L-glutaminase even in the absence of an enzyme inducer, when sea water was used as a medium. A detailed study on the molecular mechanism involved in the role of seawater components in the biosynthesis of L-glutaminase would produce information on the biology of these organisms in natural environment alongside designing an economically viable fermentation media. The effect of glutamine concentration on production was evaluated in detail, which revealed that 1% glutamine was the optimal concentration for the maximal enzyme production (Fig. 4).

A summary of purification procedure was given in Table 2. The enzyme was purified 48.12 fold. The final specific activity was 60.15. The enzyme was judged homogeneous by SDS-gel electrophoresis and the molecular weight of purified glutaminase was found to be 140 kDa (Fig. 5). L-glutaminase with molecular mass of 132 and 137 kDa was reported from *Acinetobacter glutaminasificans* and *Pseudomonas aeruginosa* respectively (Nandakumar *et al*., 2003).

IV. CONCLUSION

In conclusion, the results of the present study indicate scope for exploring marine bacterium, *B. diminuta* as a source for L-glutaminase, an enzyme that has gained industrial and pharmaceutical significance recently. Secondly marine bacteria grown in shake flasks can produce extracellular enzyme. Thirdly seawater could provide the base for fermentation media for L-glutaminase production by marine bacteria.

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