



Enhancement of *L-asparaginase* Production by *Candida utilis* in a 13 L Fermenter and its Purification

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ABSTRACT

L-asparaginase enzyme is a renowned enzyme because of its chemotherapeutic properties. This enzyme may also be employed in food processing technology. The present study aimed, optimizing the agitation and aeration rate in *L-asparaginase* production, using *C. utilis*, ATCC 9950 in batch fermentation system. Beet molasses used as the carbohydrate source for enzyme production. A maximum asparaginase activity of 245.6 U/ml obtained after 20h of fermentation under optimal condition of 1.25 vvm aeration rate and 300 rpm agitation speed. Purification of the enzyme was also done by Acetone Precipitation and column chromatograph. Purified *L-asparaginase* revealed molecular weight 40 kDa and 82.7 % yield with 10.02 fold increase in specific activity.

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1. INTRODUCTION

L-asparaginase enzyme (EC 3.5.1.1) is a known therapeutic recombinant enzyme which is used to treat some kind of blood cancers, such as acute lymphoblastic leukaemia and lymphosarcoma [1]. The discovery of asparaginase enzyme dates back to 1922, when Clementi reported the presence of the enzyme in blood of guinea pig serum [2]. After that, investigating the structure and function of this enzyme was initiated by researchers. The enzyme was produced by *E.coli* for the first time in 1964, which is marked as the beginning of biological production of asparaginase enzyme [3]. Asparaginase enzyme catalyzes the hydrolysis reaction of amino acid asparagine, which is essential for tumor cells, into aspartate and ammonia. Consequently, this function deprives the cancer cells from this amino acid [4].

The enzyme may be used in some kinds of foods because of the participation of the enzyme in catalytic

process to remove asparagine. Recent Advances in food technology have shown that cooked and fried foods (especially fried potatoes) contains a significant amount of acrylamide [5], which is caused by the reaction of reducing sugars and asparagine at temperatures above 120°C [6]. A pretreatment of potato slices with asparaginase before frying process prevents acrylamide formation [7].

Acrylamide is formed during the Maillard reaction, consisting of a series of complex reactions which occur during the heating of carbohydrates and amino acids. This reaction is mainly responsible for taste, smell, and brown color in many foods [8].

The discovery of acrylamide in food dates back to 2002 [9]. Acrylamide can cause carcinogenic effects, male fertility problems, nerve damage in humans, and weakness of immune systems which increases infectious disease and cancer [10].

L-asparaginase enzyme produced from *Aspergillus oryzae* and *Aspergillus niger* are used in baking industry to reduce acrylamide amount [11]. However, these enzymes work optimally at low temperature and don't have enough thermal stability when temperature of the

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frying process goes up to 120°C. As a consequence, stable and active enzymes over a wide range of temperature is utilizable [4]. *L-asparaginase* enzymes obtained from yeasts are thermostable [12] which makes them a positive option for food processing studies. It has been observed that eukaryotic microorganisms as yeast and filamentous fungi genera have a potential for *L-asparaginase* production with fewer adverse effects than prokaryotic microorganisms [13, 14].

Despite previous work [15] has been done by this yeast, *C. utilis* is capable of producing extraordinary asparaginase enzyme.

In the design of a fermentation process in a bioreactor, many operation parameters can influence the cell growth and enzyme activity for process scale-up. Among them, agitation and aeration rates are the most critical parameters for microorganisms in determining the productivity of the process [16].

The major goal of this study was to investigate the optimal combination condition of aeration and agitation rate to increase production of *L-asparaginase* enzyme by *C. utilis* ATCC9950 in a 13-liter laboratory bioreactor. Therefore, these two parameters variation were investigated to increase the production of the enzymes.

2. MATERIAL AND METHODS

2. 1. Microorganism *Candida utilis* ATCC9950 strain from IROST collection in Iran was used. The strains were maintained in slant at 4°C and cultured periodically.

2. 2. Chemicals

2. 2. 1. Growth Media and Inoculum Preparation

Pre-culture medium provided in a 500 ml Erlenmeyer flask containing 100 ml medium with the composition of (in grams per liter): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 20; KH₂PO₄, 6.5; K₂HPO₄, 13. The pH of the medium was 7. Inoculation was done by adding 2 ml of distilled water to slant mediums and after shaking, 0.5 ml of the supernatant was transferred to the pre-culture medium. After inoculation, the pre-culture was incubated for 16h on a rotary shaker operating at 140 rpm and 30°C.

2. 3. Bioreactor Characteristics Fermentation was carried out in the 13L stirred glass vessel bioreactor (Switzerland) with a working volume of 3L. The agitation was provided by two Pitched 4 blade (P4B) impeller (with 120 mm diameter). The bioreactor was equipped with the pH electrode, dissolve oxygen and a conductive foam sensor. The calibration of oxygen sensor arranged by air- saturated distilled water (100%),

and the pH sensor calibrated by standard buffers at pH 4 and 7 before the bioreactor sterilization. Bioreactor and the production medium sterilized separately. The sterilization of the production medium at 121°C for 15 min in an autoclave and the sterilization of bioreactor was carried out by entering the 3 bar steam pressure through the system for 30 min. Production medium needs separation of suspended compound to avoid errors in the dry weight before sterilization, This was done by centrifugation at 6000×g for 10 min.

The production medium consisted of (in grams per liter): beet molasses, 75; yeast extract, 3; KH₂PO₄, 13; NaOH, 4. The beet molasses used, contained 0.756 mg/ml total sugar. The production medium was inoculated with 0.5% (v/v) pre-culture. The temperature of fermentation process set at 30°C by circulating water in the jacket, and the pH kept at value of 7 by adding NaOH 1N. Liquid silicon was used as antifoam agent.

2. 4. Enzyme Activity The obtained *C. utilis* cells were suspended in a 20 ml buffer (pH 7), after that, the enzyme extraction was done in solution containing 20 mM 2- mercapto ethanol as a reducing agent and the solution incubated for 2h on a rotary shaker operating at 140 rpm and 50°C [15]. After this period, the solution was centrifuged to remove the cells from the crude enzyme solution.

L-asparaginase activity was assayed using heat-inactivated enzyme as a control reaction. The released aspartyl β-hydroxamate was measured spectrophotometrically at 515 nm. One unit of enzyme activity is the amount of enzyme, that liberates 1 μmol aspartyl β-hydroxamate in 1 min at 40°C [17].

2. 5. Purification of *L-Asparaginase*

2. 5. 1. Acetone Precipitation Crude enzyme extract was mixed at 4°C with cold acetone and kept at -18°C for 1 hrs. The fractionation was carried out from 30 to 80 % saturation of acetone and the highest specific activity was obtained at 60% saturation of acetone. The resultant precipitate were pelleted by centrifugation at 15,000 rpm for 15 min at 4°C. The pelleted precipitates after removing supernatant were re-suspended in Tris-HCl buffer (pH 7.2) to same volume of the starting volume, Then the protein content (at 280 nm) and enzyme activity was measured.

2.5. 2. Purification by Coulum Chromotogeraph Anion-exchange chromatography was carried out using Q-sepharose. Q-sepharose column was equilibrated with 0.01 M Tris-HCl buffer (pH 7.2) and enzyme solution was adsorbed on the column. After the column was washed thoroughly with the same buffer according to the method of sakamoto [18], step-wise elution with the

buffer containing NaCl was done for the purpose of eliminating carbohydrates.

2. 6. Disc-electrophoresis SDS-PAGE was done according to the method of Laemmli [19], with a separating acrylamide gel of 10% and stacking gel 5% containing 0.1% SDS. The gel was stained with coomassie brilliant blue R-250 and destained with a solution of methanol, acetic acid and water in the ratio of 4:1:5. The molecular weight was determined by SDS – PAGE of proteins of known molecular weight along with the protein to be characterized .

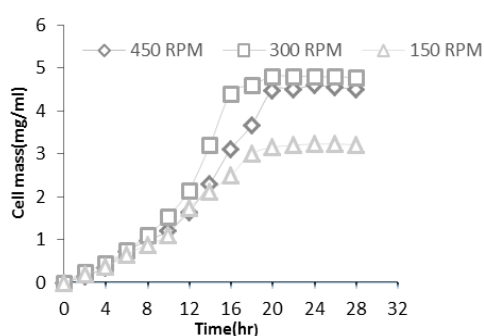


Figure 1. The effect of different agitation speeds on cell mass production

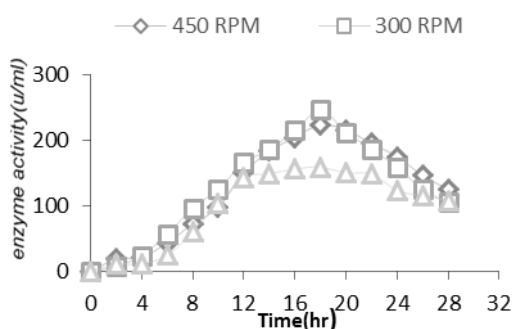


Figure 2. The effect of different agitation speeds on enzyme activity

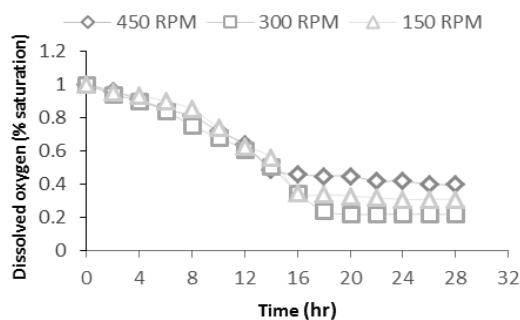


Figure 3. The effect of agitation speeds on dissolved oxygen

3. RESULTS AND DISCUSSION

Experiments were conducted at agitation speeds of 150, 300, and 450 rpm with enough aeration rate of 1.25 vvm. After that, the agitation speed was kept at 300 rpm (optimized value) and the aeration rate was changed to 0.5, 1.25, and 2 vvm. In all experiments, samples were drawn at exact intervals and analyzed for biomass, total sugar and enzyme activity.

3. 1. Effect of Agitation Speed Agitation speeds improves the mass transfer of oxygen from the gas bubbles from the environment and can influence on dissolved oxygen of medium [20]. However, at higher agitation speeds, shearing forces are increased which causes improvement in growth of microorganisms and production of some useful products as enzymes. To see the effect of increase in agitation speed, three different stirring speeds for 28 h at 30 ° C was studied (Figures 1 to 3).

Increasing the agitation speed from 150 to 300 rpm proved to be beneficial for the growth of microorganisms and enzyme production (Figures 1 and 2). As can be seen in Figures 1 and 2, after 20h of fermentation time, the rate of changes in the cells mass was very low and the growth rate remained relatively constant. Maximum cell mass was observed at 20h and 300 rpm (4.81 mg/ml), and the maximum enzyme activity was obtained (245.6 IU/ml) at 18h and 300 rpm. The cells mass and enzyme activity reduced when the agitation speed increased to 450 rpm (223.2 IU/ml, 4.66 mg/ml).

Oxygen concentration profiles of the three agitation speeds are shown in Figure 3. Dissolved oxygen concentration for all agitation speeds significantly reduced up to 18h of fermentation time. However, after that, this reduction was low and in 300 rpm even remained constant on 22%, which reflects the beginning of stationary phase in growth process of microorganisms. The results obtained, indicate that the cultivation at moderate agitation speed was better for production of asparaginase enzyme and cell growth. This result is in agreement with other investigator [21].

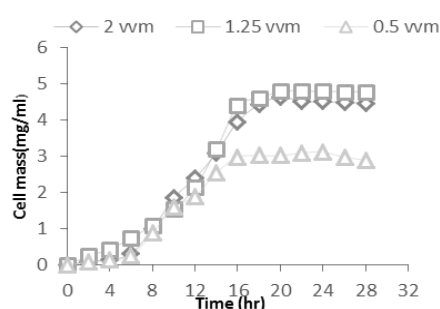


Figure 4. The effect of aeration rates on cell mass production

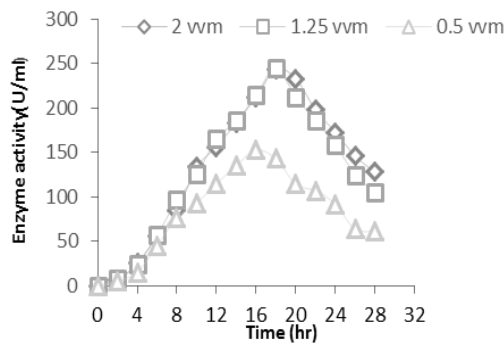


Figure 5. The effect of aeration rates on enzyme activity

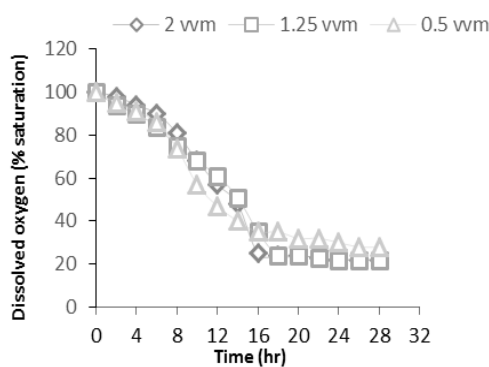


Figure 6. The effect of aeration rates on dissolved oxygen

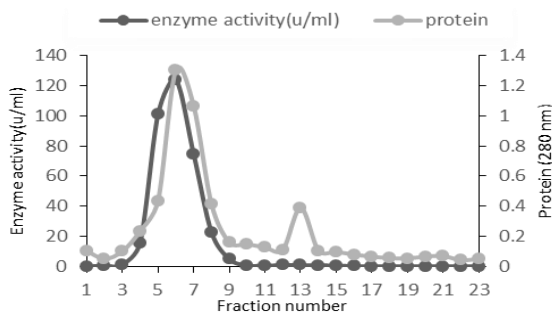


Figure 7. Q-sepharose ion exchange chromatography of *L-Asparaginase* showing enzyme activity and protein content profile.

TABLE 1. Purification of *L-asparaginase*

| Sample | Protein content (mg/ml) | Enzyme activity (U/ml) | Specific activity (U/mg) | Fold | Recovery (%) |
|--------------------|-------------------------|------------------------|--------------------------|-------|--------------|
| Crude | 0.187 | 146.57 | 783.79 | ----- | 100 |
| 60% acetone | 0.0175 | 122.48 | 6998.8 | 8.93 | 83.5 |
| Q-sepharose column | 0.0155 | 121.3 | 7853 | 10.02 | 82.7 |

3. 2. Effect of Aeration Rate In the present study, *L-asparaginase* production by *C. utilis* is considered under different levels of aeration rate (0.5 – 2 vvm) at 300 rpm (optimized agitation speed) presented in Figures 4-6. Increase in biomass was similar for all studied aeration rates until a certain time (18h), after that, a negligible increase in cell mass was observed at 1.5 and 2 vvm. Even at 0.5 vvm aeration rate, the cell mass reduced that gets along to insufficient oxygen level and death of a number of cells. Maximum growth of *C. utilis* (4.81 mg/ml) and the highest amount of enzyme production (245.6 IU/ml) observed after 18h of fermentation time at 1.25 vvm aeration rate. The highest amount of enzyme production for other aeration rates (159 and 244.3 IU/ml) was observed at 0.5 and 2 vvm. These results indicate that aeration rate has a significant role on *L-asparaginase* enzyme production and the growth of *C. utilis* ATCC 9950 cells were found to be affected by the supply of oxygen during the course of fermentation. By increasing the aeration rate from 1.25 to 2 vvm, no significant changes in enzyme production was observed and it was reduced to 244.3 IU/ml. Comparison of Figures 4 and 5 shows the growth - dependent behavior of this microorganism. Dissolved oxygen varied considerably under various aeration rates and were different at the end of fermentation time (Figure 6). The lowest dissolved oxygen concentration was observed at 1.25 and 2 vvm aeration rate (22%). Similar results of aeration rate effect on cell growth obtained by other researchers. Maximum cell growth has been observed at moderate aeration rate for microbial systems like *E. aerogenes* and *E. cartovora* [21, 22]. In addition, higher aeration rates has not significant influence on enzyme production [23].

3. 3. Purification The results for purification of *L- asparaginase* are summarized in Table 1. The specific activity of the crude extract was 783.79 units/ mg protein. 60% saturation of acetone fractionation showed 8.93 fold purification of enzyme with 83.5% recovery (Table 1). *L-asparaginase* from other microorganisms i.e. [24], [25] also showed maximum activity in same fraction. Activity was increased by 10.02 fold after gel permeation chromatography using Q-sepharose column, though enzyme recovery was reduced to 82.7%. Maximum enzyme activity is shown by 6th and 7th fraction of gel permeation (Figure 7) and pooled out.

Electrophoresis study reveals that single band in Third lane shows the purified enzyme (Figure 8). Approximate molecular weight of purified enzyme was found to be 40 kDa, as determined by comparing with it against protein marker ladder on gel. Manna et al., [26] reported the molecular weight of *L-asparaginase* as 34kDa which is comparable to present study. In one of their study, Moorthy et al. purified the *L-asparaginase* from bacillus. sp and reported that its molecular weight was 45 kDa .

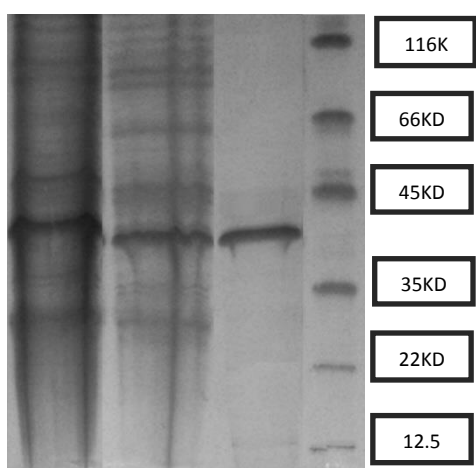


Figure 8. The result of PAGE. In first lane crude enzyme, second lane has fraction of acetone precipitation, third lane has a single band of fraction of Q-sepharose column. Forth lane has broad range protein marker.

4. CONCLUSIONS

L-asparaginase enzyme is obtained from different microbial sources which almost of these enzymes have been produced with the aim of therapeutic treatment. In the present study, because of the properties of the asparaginase enzyme in the elimination of acrylamide in some kinds of foods containing carbohydrate and asparagine aminoacid, optimization of *L-asparaginase* production was studied utilizable in food processing. It is hoped that due to the growing recognition of international society from acrylamide as a carcinogenic material, the utilization of this enzyme becomes widespread in the food industry.

The Present study indicates that influence of aeration rate on asparaginase production from *C. utilis* is significant. Aeration rate of 1.25 vvm is the optimal aeration rate for production of the enzyme. Higher agitation speeds, more than 150 rpm have a significant impact on *L-asparaginase* production and cell mass. Higher agitation speeds greater than 300 rpm decreases the enzyme activity and this can be due to shearing forces.

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آنزیم ال آسپارژیناز به علت خصوصیات درمانی اش آنزیم بسیار شناخته شده ای می باشد. این آنزیم می تواند در فرآوری مواد غذایی نیز استفاده شود. مطالعه اخیر با هدف بهینه سازی هوادهی و همزدن در تولید آنزیم آسپارژیناز با استفاده از مخمر کاندیدا یوتیلیس ATCC 9950 در سیستم تخمیری ناپیوسته انجام شده است. ملاس چغندر قند به عنوان منبع کربوهیدرات در تولید آنزیم استفاده شده است. بیشترین فعالیت آنزیم تولیدی پس از 20 ساعت تخمیر تحت هوادهی 1/25 vvm و همزدن 300 rpm به دست آمد. در انتها تخلیص این آنزیم به وسیله رسوب با استون و ستون کروماتوگرافی نیز انجام شده است. آنزیم آسپارژیناز تخلیص شده وزن ملکولی 40 KDa کیلودالتون داشته و نسبت تخلیص و درصد بازیافت آنزیم به ترتیب به 10/2 و 82/7٪ افزایش یافت.

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