



## BENCH-SCALE PRODUCTION OF L-ASPARAGINASE FROM *ERWINIA CAROTOVORA* IN A LABORATORY FERMENTER

**SARITA DEVI, ADITYA KULSHRESHTHA, ASHUTOSH KUMAR RAI AND WAMIK AZMI\***

**Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla Himachal Pradesh-171005 India**

### ABSTRACT

The discovery that L-asparaginase acts as an antitumor agent in children with lymphoblastic leukemia led to the extensive studies on the physicochemical properties of the enzyme as well as on clinical effect. In the present study, attempts were made to optimize various culture parameters for the production of L-asparaginase by *Erwinia caratovora* MTCC 1428 in a bench scale fermenter. Different sets of physical parameters were designed to enhance the production of L-asparaginase at minimum cost and time. The optimized condition for maximum production of cell mass of *E. caratovora* MTCC 1428 and maximum L-asparaginase activity obtained were 300 rpm and 0.5 vvm, respectively without pH control. The maximum L-asparaginase activity and maximum cell mass of *E. caratovora* MTCC 1428 achieved during this fermentation studies was 0.176 U/mg dcw and 1.465 mg/mL at 8 h and 18 h, respectively. The biosynthesis of L-asparaginase was increased 1.98 fold in optimized physical parameters.

**Keywords:** Acute lymphoblastic leukemia, *Erwinia caratovora* MTCC 1428, L-asparaginase, fermentation, aeration, dissolved oxygen, agitation.

### INTRODUCTION

L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) is an anti-neoplastic agent used in lymphoblastic leukaemia chemotherapy [Gokbuget and Holzer, 2002; Narta *et al.*, 2007; Narayana *et al.*, 2008]. L-asparaginase is a hydrolytic enzyme which carries out cleavage of L-asparagine into L-aspartic acid and ammonia and it belongs to amidase group and this catalytic reaction is essentially irreversible under physiological conditions [Lubkowski *et al.*, 1996; Balkao *et al.*, 2001; Prakasham *et al.*, 2007; Sarquis *et al.*, 2004]. L-asparagine acts as an essential amino acid for the growth of tumor cells. They require an external input of this amino acid whereas the normal cells are independent of its requirement because they can synthesize L-asparagine by the enzyme L-asparagine synthetase [Narta *et al.*, 2007]. When the L-asparaginase is provided to the tumor cells it causes the

deprivation of the cells, as an important growth factor becomes unavailable to them and the cells cannot survive any more. This fact suggests that the enzyme can be used as an antitumor drug. The precise mechanism of L-asparaginase action is still unknown, although hydrolysis is known to proceed in two steps via a beta-acyl-enzyme intermediate [Narta *et al.*, 2007; Moola *et al.*, 1994; Manna *et al.*, 1995]. Unlike other chemotherapy agents, it can be given as an intramuscular, subcutaneous, or intravenous injection without fear of tissue irritation [Gokbuget and Holzer, 2002; Narta *et al.*, 2007; Narayana *et al.*, 2008]. It is marketed under the brand name *Elspar*, to treat acute lymphoblastic leukemia [Narta *et al.*, 2007].

Using amino acid sequences and biochemical properties as criteria, enzymes with asparaginase activity can be divided into several

families [Borek and Jaskolski, 2001]. The two largest and best-characterized families include bacterial and plant-type asparaginases. The bacterial-type enzymes have been studied for over 40 years [Ortlund *et al.*, 2000]; their homologs are found in some mammals and in fungi. In particular, enzymes such as glutamin-(asparagin)-ases [Ortlund *et al.*, 2000], lysophospholipases [Sugimoto *et al.*, 1998], and the  $\alpha$ -subunit of GlutRNA amidotransferase [Curnow *et al.*, 1998] can also be considered part of the bacterial asparaginase family. It has been shown on the basis of kinetic and structural studies that two conserved amino acid motifs are responsible for the activity of the above mentioned proteins [Lubkowski *et al.*, 2003; Oza *et al.*, 2009].

L-Asparaginase has been isolated from a number of sources. These include *Proteus vulgaris* [Cammack *et al.*, 1972], *Serratia marcescens* [Whelan and Wriston, 1969], *Streptomyces griseus* [De-Jong, 1972], *Achromobacteraceae* [Roberts *et al.*, 1972], and *Pisum sativum L.* [Sieciechowicz *et al.*, 1988]. Though L-asparaginase has been reported in many higher plants, little work has been carried out on the characterization of L-asparaginase from higher plants. The presence of an amidase in barley roots capable of hydrolyzing L-asparagine had been reported earlier, and the distribution of L-asparaginase in *Lupinus luteus* and *Dolichos lablab* seedlings has also been reported [Oza *et al.*, 2009].

Now preparations based on periplasma asparaginase from wild strains of *E. coli* and *Erwinia Chrysanthemi* are used in clinical practice for treatment of acute lymphoblast leukemias, acute myeloblast leukemias, myelomas, and various lymphomas [Abakumova *et al.*, 2009; Perel *et al.*, 2002]. However, due to the prolonged administration of L-asparaginase, the corresponding antibodies are produced in man, which causes an anaphylactic shock or neutralization of the drug effect [Amena *et al.*, 2010]. Therefore there is a continuing need to screen newer organisms in order to obtain strains capable of producing new and high yield of L-asparaginase.

This paper describes the effect of various physical parameters used for growing *Erwinia carotovora* MTCC 1428 with high enzyme production with minimum cost and time in a bench scale fermenter.

## MATERIALS AND METHODS

### 1. Chemicals

The chemicals used in the experiments were procured from Himedia, Merck and SDfine, India and were of analytical grade.

### 2. Microorganism and maintenance of culture

The culture of *E. caratovora* MTCC 1428 was procured from Department of Biotechnology, Himachal Pradesh University, Shimla-5. The culture of *E. caratovora* MTCC 1428 was maintained on medium containing (% w/v) casein enzyme hydrolysate (tryptone) 1.5, peptone 0.5, NaCl 0.5 and agar 2.0 (pH 7.0) slants containing 1.0% (w/v) L-asparagine or in 20% (v/v) glycerol stocks and sub culturing was done periodically with the same medium at 25°C. Phenol Red (0.0012%) was used as an indicator for asparaginase activity. The production of L-asparaginase was generally accompanied by increase in pH due to formation of ammonia after the hydrolysis of L-asparagine. Phenol Red at acidic pH was yellow and turned pink at alkaline pH. Thus a pink zone was formed around the colonies of *E. caratovora* MTCC 1428.

### 3. Inoculum and medium preparation for bench scale fermentation

The optimum conditions for production of cell mass and L-asparaginase activity by fermentation at a scale of 6 L were investigated for *E. caratovora* MTCC 1428. The medium components and different physical parameters were optimized in shake flask level and the optimized parameters were used for bench scale fermentation also (data not shown). For the development of a laboratory inoculum, the seed was prepared in conical flasks containing the seed medium (% w/v; tryptone 1.0, peptone 1.0 and L-asparagine 0.1). The seed medium was inoculated with organism *E. caratovora* MTCC 1428, and incubated at 25°C for 12 h on a rotary shaker (150 rpm). The production medium (pH 7.0) contained (% w/v) tryptone 1.0, peptone 1.0 and L-asparagine 0.6. The fermenter was loaded with production medium with additionally contained 0.01% (v/v) silicone oil as antifoam agent (Himedia). The medium was sterilized *in situ* with the help of steam supplied through the jacket of fermenter.

#### **4. L-asparaginase assay**

L-asparaginase catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia and the amount of liberated ammonia was measured spectrophotometrically. The L-asparaginase from *E. caratovora* MTCC 1428 was found to be intracellular in nature and hence the resting cells suspended in potassium phosphate buffer (0.05 M, pH 8.5) were used for the enzyme assay. Potassium phosphate buffer 1.45mL (0.05 M, pH 8.5), cell suspension (50  $\mu$ L) of known dew and 500  $\mu$ L of 10 mM substrate (L-asparagine) prepared in potassium phosphate buffer were incubated at 37°C for 15 min. The reaction was stopped by the addition of 500  $\mu$ L chilled TCA (15 %, w/v). A set of control was also run. From the reaction mixture, 1 mL was withdrawn and amount of released ammonia was measured by ammonia hypochloride method [Fawcett and Scott, 1960]. Activity of the L-asparaginase from the whole cell of *E. caratovora* MTCC 1428 was expressed in terms of units (U). The L-asparaginase unit has been defined as the  $\mu$ moles of ammonia released by one mg of dry cell weight in one minute under standard assay condition.

#### **5. Sterilization and inoculation of the production medium**

The fermentation was carried out in 14 L laboratory fermenter (BIOFERM-LS2, Scigenics India Pvt. Ltd.) at 6 L working volume in batch mode. As the fermenter has inbuilt facility of sterilization (*in situ* sterilizable), all the ingredients of the production medium (%), w/v; tryptone 1.0, peptone 1.0 and L-asparagine 0.6 were dissolved in distilled water, and the 6 L production medium was loaded into the fermenter. The sterilization of the production medium was carried out at 121°C for 15 min. After the completion of sterilization cycle, the temperature of the medium was adjusted to 25°C by cooling. The sterilized production medium was inoculated with 4% (v/v), 12 h old seed culture through the inoculation port by peristaltic pump attached to a feed bottle. The pH, temperature and dissolved oxygen were monitored throughout the operation. However, temperature was maintained at 25°C for the entire course of fermentation with the help of inbuilt heater and chiller.

#### **6. Growth and Production of L-asparaginase**

The growth of *E. caratovora* MTCC 1428 cells and activity of L-asparaginase was measured under different conditions of agitation, aeration and pH by adopting the analytical procedures. The effect of these variables on pH, dissolved oxygen (DO, % saturation), cell mass and L-asparaginase activity was observed.

#### **7. Effect of agitation on the growth and production of L-asparaginase activity by *E. caratovora* MTCC 1428**

The effects of agitation on production of biomass, L-asparaginase activity, DO and pH by *E. caratovora* MTCC 1428 was investigated by varying the agitation rate (150, 300 and 450 rpm). The fermentation was carried out at 25°C temperature keeping the aeration rate 0.5 vvm. Samples at regular interval of 2 h were withdrawn and analyzed. The pH and DO of the fermentation broth during the entire course of cultivation were monitored with the help of DO and pH probe. The suitability of the agitation rate was determined on the basis of the results obtained.

#### **8. Effect of aeration rate on growth and production of L-asparaginase by *E. caratovora* MTCC 1428**

The effect of aeration rate on the growth and production of L-asparaginase by *E. caratovora* MTCC 1428 and change in pH and DO profile of the fermentation broth was investigated under varying aeration rate (0.25 to 0.75 vvm) at 300 rpm.

#### **9. Effect of pH on growth and production of L-asparaginase by *E. caratovora* MTCC 1428**

The crucial effect of pH on the production of L-asparaginase was observed during the experiments in shake flask level (data not shown), hence it become necessary to study the effect of pH on the growth and production of L-asparaginase by *E. caratovora* MTCC 1428 in bench scale fermentation. The previous experiments of agitational speed and aeration rate optimization were carried out at initial medium pH of 7.0 without any further control. However, in the next experiment, the pH of the medium was controlled at 7.0 with the help of acid (4 N HCl) through automated pumping by peristaltic pumps. The effect of controlled pH on cell growth, production of L-asparaginase and DO profile of the medium

was investigated under the condition of 0.5 vvm aeration, 300 rpm agitation and at 25°C temperature.

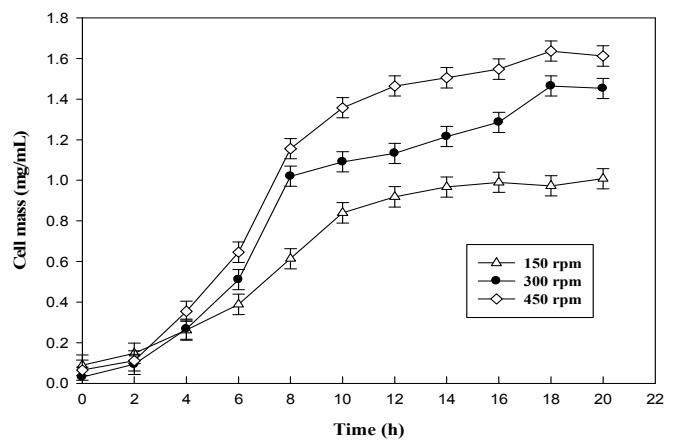
## RESULTS AND DISCUSSIONS

The fermenter was well equipped with pH, temperature, agitation, aeration, and dissolved oxygen sensors and controls. In the present study, the effect of aeration rate, agitation rate and pH on cell growth, L-asparaginase production and other parameters such as pH, DO were determined during the course of fermentation of *E. carotovora* MTCC 1428.

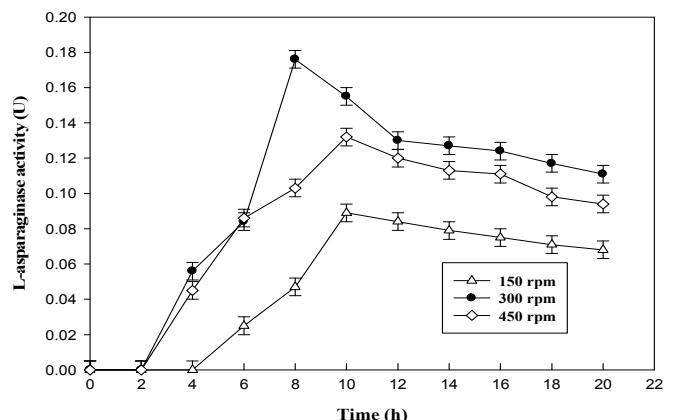
The agitation improves the rate of oxygen transfer from bulk gas to the medium, which has ultimately been utilized by the growing microorganism. It also ensures the recycling of the nutrient pool to the growing microorganism and thereby resulting in the better production of L-asparaginase. However, at higher agitation the shearing forces also become operative and sometimes prove to be retarding both for growth as well as the production of some useful products. The fermentation was carried out at controlled temperature of 25°C with optimized medium containing (% w/v) tryptone 1.0, peptone 1.0 and L-asparagine 0.6 at 0.5 vvm aeration rate but at different agitation speed (150, 300 and 450 rpm). The composition of seed medium was same as production medium except L-asparagine concentration (0.1%, w/v). The fermentation was carried out for 20 h in batch mode. The increase in the agitation speed from 150 rpm to 450 rpm proved to be beneficial for the growth of the *E. carotovora* MTCC 1428 cells. The cell mass of 1.64 mg/mL was obtained at 18<sup>th</sup> h with 450 rpm, which was higher than the cell mass attained

under 300 rpm at the same time period (Fig. 1 a). However, at 150 rpm agitation the growth rate was slower and this caused an early attainment of the stationary phase. So the 450 rpm of agitation speed at 0.5 vvm aeration was found to be the most optimum for the growth of *E. carotovora* MTCC 1428. The optimum combination of rate of agitation and aeration was essential for the hyper production of L-asparaginase. The maximum enzyme activity of 0.176 U was obtained in 8<sup>th</sup> h at 300 rpm agitation (Fig. 1 b) followed by at agitation rate of 450. In case of 150 rpm, the maximum enzyme activity was found to be 0.089 U at 10<sup>th</sup> h of fermentation.

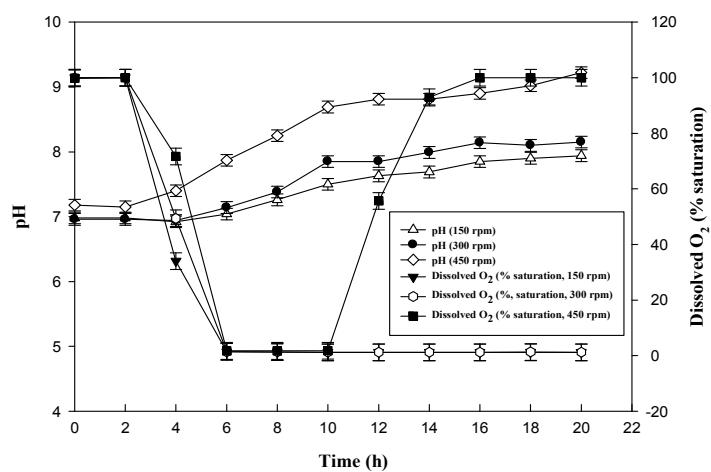
Although the agitation has more profound effect in improving the dissolved oxygen concentration than aeration, some negative factors were also associated with the agitation speed that can hamper the net benefit an aerobe can have. An increase in the agitation rate beyond 300 rpm proved to be ineffective in enhancing the L-asparaginase production, and in fact resulted in decrease in L-asparaginase activity. This decline may be attributed to the effect of shearing forces that become operative at higher speed of stirrer. The dissolved oxygen profiles of the fermentation broth under different rates of agitation reveals that depletion in the dissolved oxygen was insignificant at the higher rate of agitation (Fig. 1 c). In contrast to the decline in DO from 100% to 1.4% during first 6 h of the course of fermentation at 150 rpm and 300 rpm, the DO level at higher agitation rate (450 rpm) first dropped below 1.7% during first 6 h and then started to increase from 10 h and reached to 100% at 16<sup>th</sup> h of fermentation. The agitation speed obtained for production of L-asparaginase from *S. gulbargensis* was 200 rpm [Amena *et al.*, 2010].



(a)



(b)



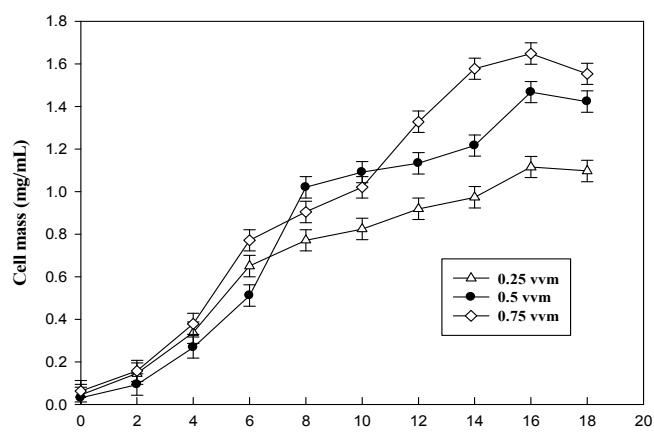
(c)

**Fig. 1 Effect of agitation rate on (a) cell mass production, (b) L-asparaginase activity and (c) pH and dissolved oxygen by *E. carotovora* MTCC 1428 in laboratory fermenter**

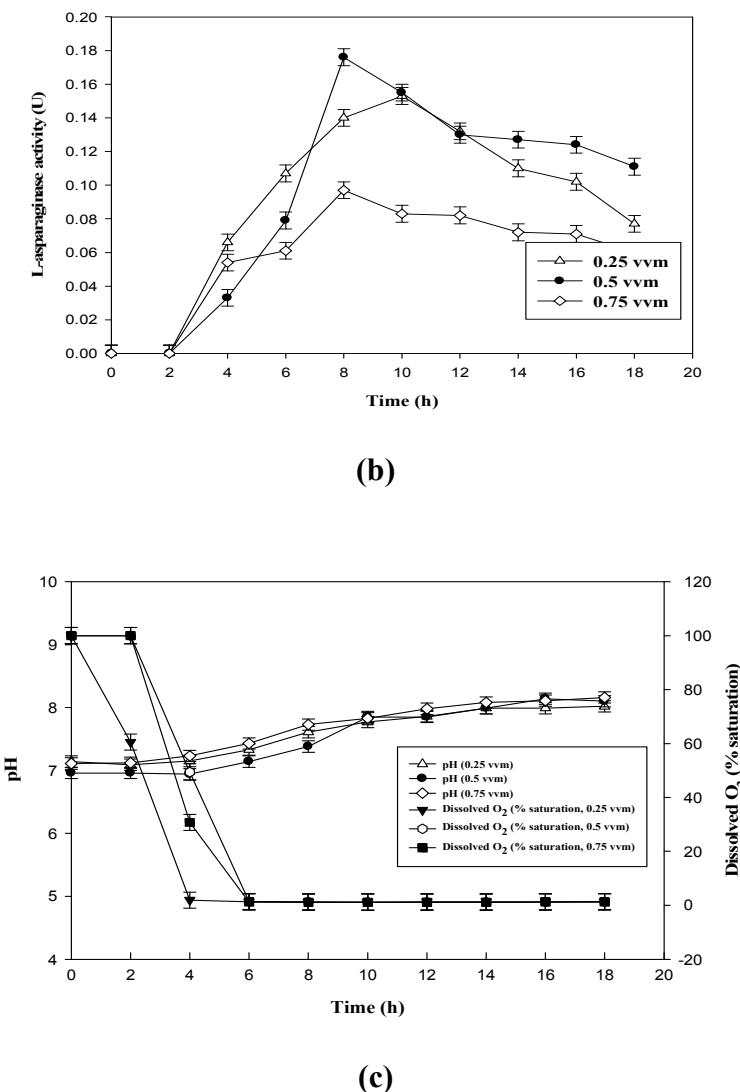
The pH of the fermentation broth was also found to increase during the course of fermentation in all the experiments. It can be inferred from these results that rate of agitation was helpful in maintaining the higher dissolved oxygen level which subsequently help in growth and L-asparaginase production. The fermentation kinetics of *E. aerogenes* in a 2 L bioreactor for the production of L-asparaginase suggest the beneficial effect of higher rate of agitation (700 rpm) at 1 vvm aeration for the production of L-asparaginase [Mukherjee *et al.*, 2000]. However, certain other organisms have been reported to produce L-asparaginase only under oxygen deficient conditions notwithstanding their better growth under aerobic conditions [Liu and Zajic 1973; Bascomb *et al.*, 1975]. Another report reveals the inhibitory role of dissolved oxygen in the medium on the synthesis of L-asparaginase by *E. coli* A-1 [Barnes *et al.*, 1977]. The biosynthesis of L-asparaginase occurred during the period of limited oxygen availability. After the return of dissolved oxygen to the fermentation medium at 39 h, both the number of viable cells and the enzyme levels decreased for *S. marcescens* [Heinemann and Howard, 1969]. The course of fermentation was also studied in case of the production of the L-asparaginase using agricultural waste for *A. niger* [Mishra, 2006]. The production of enzyme appeared growth

dependent and a 96 h of fermentation time supported maximum yield by *A. niger*. The optimum L-asparaginase production was obtained in 3% nutrient broth and 1% MSG medium when the pH was maintained at 7.5 and the dissolved oxygen level was at 0% in case of *E. coli* [Barnes *et al.*, 1978].

In the present study, the culture *E. carotovora* MTCC 1428 has been considered as a facultative anaerobe and hence these cells were grown at varying aeration rate (0.25-0.75 vvm) at the agitation speed of 300 rpm. The growth of *E. carotovora* MTCC 1428 cells were found to be affected by the supply of oxygen during the course of fermentation. The maximum growth of *E. carotovora* MTCC 1428 was obtained at 16<sup>th</sup> h of fermentation (1.65 mg/mL) at 0.75 vvm aeration rate. The cell mass production increased up to 16<sup>th</sup> h of incubation and after that it started decreasing at 0.75 vvm aeration (Fig. 2 a). However, the maximum biomass (1.47 mg/mL) at 0.5 vvm aeration rate was also obtained at 16<sup>th</sup> h of fermentation. The maximum L-asparaginase production (0.176 U) was observed at 8<sup>th</sup> h of fermentation at 0.5 vvm aeration followed by 0.155 U at 8<sup>th</sup> h at 0.25 vvm (Fig. 2 b). This might be due to the inhibitory effect of the dissolved oxygen concentration during the course of fermentation for L-asparaginase production by *E. carotovora* MTCC 1428 cells.



(a)



**Fig. 2 Effect of aeration rate on (a) cell mass production, (b) L-asparaginase activity and (c) pH and dissolved oxygen by *E. carotovora* MTCC 1428 in fermenter**

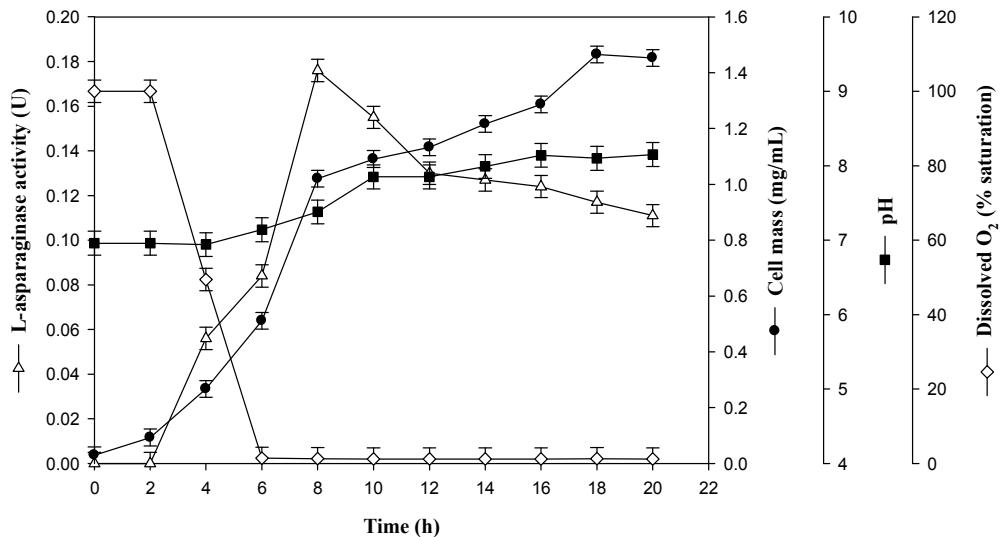
The L-asparaginase activity was reported to be 960 IU/g at 0.5 vvm aeration rate for *E. aroideae* [Peterson and Ciegler, 1969]. A stimulatory effect of aeration on cell growth has also been observed by other authors as well, in other microbial systems like *E. aerogenes* and *Citrobacter* [Bascomb *et al.*, 1975; Mukherjee *et al.*, 2000]. However, some workers have also reported the maximum production of L-asparaginase from *E. aroideae* and *S. marcescens* at an anaerobic condition [Heinemann *et al.*, 1970]. Apart from the effect of oxygen supply on the growth and production of L-asparaginase, its effect on final pH and dissolved oxygen consumption of fermentation broth was also studied. The final pH and dissolved oxygen profile in all three cases showed similar pattern

(Fig. 2 c). There was drastic decrease in dissolved oxygen consumption at first 6 h after that it remained constant at 1.4% throughout the course of fermentation.

There was an increase in pH in all the cases and the pH of the fermentation broth reached up to 8.1. The maximum L-asparaginase production from *P. vulgaris* was obtained when 1 QAR (millimole of O<sub>2</sub> absorbed per litre of culture medium per minute) was used as aeration rate during fermentation [Tosa *et al.*, 1972]. During the present study 0.5 vvm aeration at 300 rpm agitation speed was found to be the most optimum for the production of L-asparaginase by *E. carotovora* MTCC 1428 in a laboratory fermenter. The fermentation by *E. carotovora* MTCC 1428 cells for L-asparaginase production

was carried out in the optimized medium (pH 7.0) without pH control at 0.5 vvm aeration and 300 rpm agitation speed. The maximum enzyme activity obtained was 0.176 U at 8 h of incubation and the maximum cell mass (1.47 mg/mL) of *E. carotovora* MTCC 1428 was

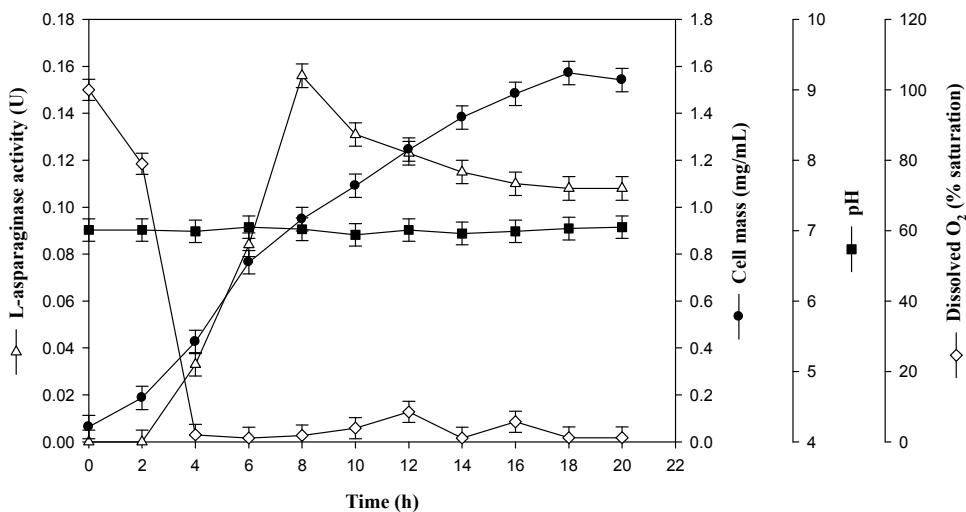
observed at 18 h of fermentation (Fig. 3). The increase in cell mass of *E. carotovora* MTCC 1428 leads to the rapid utilization of oxygen which reaches to minimum at 6 h of fermentation, and thereafter that concentration of dissolved oxygen was almost around zero.



**Fig. 3 The course of fermentation by *E. carotovora* MTCC 1428 cells for the production of L-asparaginase without pH control**

The pH of the fermentation broth increased continuously due to the liberation of ammonia and this increase in the pH found to be associated with the decrease in L-asparaginase activity. Heinemann *et al.*, [1970] studied the changes in pH during production of L-asparaginase by *S. marcescens* in 55 L scale fermenter. The optimum pH for the production of L-asparaginase by *E. carotovora* MTCC 1428 was found to be 7.0 in shake flask studies. The same pH was maintained throughout the fermentation with the help of pH controller by using HCl (4 N) for the growth and L-asparaginase production by *E. carotovora* MTCC 1428. The agitation rate was fixed at 300 rpm at 0.5 vvm aeration rate. The maximum (0.156 U) L-asparaginase activity was found at 8 h of fermentation (Fig. 4). The dissolved oxygen concentration dropped to 1.2% drastically between 3-4 h and remained constant throughout the course of fermentation. The maximum cell mass of *E. carotovora* MTCC 1428 was obtained at 18 h (1.57 mg/mL). These results suggest that the pH control does not have much role on L-

asparaginase production by *E. carotovora* MTCC 1428. The maximum enzyme activity of 0.1 IU/mg dw was reported in *E. coli* R-26 and maximum production of cell mass was 1.80 mg/mL at controlled pH of 7.5 [Barnes *et al.*, 1978]. The pH was also controlled during the production of L-asparaginase from a strain of *E. coli* B by Boeck *et al.*, [1970]. The increase in final pH was also observed in *S. albidoflavus* by Narayana *et al.*, [2008]. Amena *et al.*, [2010] have reported the optimum pH for L-asparaginase production by *S. gulbargensis* to be 6.5. The production of *E. carotovora* MTCC 1428 cells with high L-asparaginase activity was found to be stimulated by supply of the complex medium components. Moreover, their production has also been affected by the physico-chemical as well as other fermentation parameters. The study of agitation and aeration rate in a bench scale fermenter result in the increase in L-asparaginase activity (from 0.126 to 0.176 U) and reduction in fermentation time of *E. carotovora* MTCC 1428 from 14 to 8 h.



**Fig. 4 The course of fermentation by *E. carotovora* MTCC 1428 cells for the production of L-asparaginase with pH control**

## CONCLUSION

The L-asparaginases from different sources possess different immunological complications due to the presence of glutaminase. *E. carotovora* L-asparaginase may represent an important alternative therapy as a patient develops hypersensitivity to the other enzymes. The L-asparaginases from *E. carotovora* MTCC 1428 is glutaminase free and this property of L-asparaginase represents an important strategy for the creation of next-generation therapeutic products with enhanced pharmacological possessions. The production of this enzyme has a broader prospectus in industrial area and even in pharmaceutical industries as the microbial production of L-asparaginase is inexpensive. This

process can be amenable to large scale production and may be of interest to researchers and biopharmaceutical companies interested in developing and improving their therapeutic properties, which offer a great opportunity to scientific, biotechnological, economical, and industrial growth.

## ACKNOWLEDGEMENTS

The research was supported by University Grants commission, Government of India, New Delhi, India. The authors wish to acknowledge the internet facility provided by Sub-Distributed Information Centre, Department of Biotechnology, Himachal Pradesh University, Shimla-5, India.

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