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Production of Nitrilase by a Recombinant *Escherichia coli* in a Laboratory Scale Bioreactor

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Abstract

Effects of medium pH (uncontrolled and controlled), aeration rate and agitation intensity on the production of biomass and nitrilase by a recombinant *Escherichia coli* in a stirred-tank bioreactor are reported. The recombinant bacterium expressed the nitrilase gene of *Alcaligenes faecalis*. The initial pH of the culture medium had a strong influence on the growth of biomass and enzyme production. In batch fermentation process the growth and enzyme production were maximized at 37°C with an initial medium pH 7.0. The fermentation was influenced by oxygen transfer efficiency of the bioreactor and by the turbulence regimen. The optimal production conditions were an aeration rate of 0.4 vvm and an agitation speed of 400 rpm. Higher values of agitation speed and aeration rate proved detrimental to both biomass production and nitrilase activity. Under optimal conditions, the final dry biomass concentration was 6.9 g/L and the biomass specific enzyme activity was 58 U/g dry cells.

Keywords: Nitrilase; Mandelic acid; Mandelonitrile; Fermentation; Recombinant *Escherichia coli*; *Alcaligenes faecalis*

Introduction

The enzyme nitrilase (EC 3.5.5.1) catalyzes the hydrolysis of nitrile (–CN) functional groups in various compounds to the corresponding carboxylic acids and ammonia [1,2]. Nitrilase-mediated biotransformations have been extensively reviewed in the literature [2-5]. The *enantio*- and *regio*-selectivity of nitrilases offer synthetic possibilities that are difficult to achieve by non-enzymatic catalysis [6]. Production of enantiomerically pure (*R*)-(–)-mandelic acid by nitrilase-catalyzed conversion of mandelonitrile is well known [7-10]. Immobilization of nitrilases in the form of cross linked enzyme aggregates (CLEAs) has been reported [11,12].

Nitrilases from several microbial sources have been purified, characterized and used in biotransformations [2-5,10,13-15] and reports were also available for enhancing the production of nitrilases by optimizing the fermentation process parameters [6,16-18]. Titers of many other enzymes have been shown to be highly dependent on the fermentation conditions used in their production [19-21].

This work is focused on production of intracellular nitrilase in a recombinant *Escherichia coli* expressing the nitrilase gene of *Alcaligenes faecalis* [11]. Earlier studies have discussed the gene cloning of nitrilases of *Pseudomonas putida* [16] and *Pseudomonas fluorescens*[13]. The nitrilase gene of *Rhodococcus rhodochrous* has also been cloned and overexpressed in *E. coli* [14]. Cloning, production and properties of a highly thermostable and enantioselective nitrilase of an *Alcaligenes* sp. have been described in literature [10,15]. This paper mainly focused on the interaction of agitation and aeration in a laboratory scale bioreactor during the growth of *E. coli* for the production of nitrilase.

Materials and Methods

Chemicals

Mandelonitrile, L-rhamnose and polypropylene glycol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Growth media components were obtained from Hi-media Inc. (Mumbai, India).

Microorganism and cultivation conditions

A recombinant *E. coli* JM109 expressing the nitrilase gene of *Alcaligenes faecalis* MTCC-126 [11] was used. The stock culture was maintained at 4°C on plates of Luria Bertani (LB) agar supplemented with ampicillin (100 µg/mL). A loopfull of the microorganism from the stock culture was used to aseptically inoculate 50 mL of LB broth (supplemented with 100 µg/mL of ampicillin) in a 250 mL shake flask [17]. After 16 h (37°C, 200 rpm), the above culture (50 mL) was used to inoculate 500 mL of LB broth (supplemented with 100 µg/mL ampicillin). In the second stage of inoculum preparation, L-rhamnose (2 g/L) was added as an inducer after 4 h of incubation of the inoculum.

The above preculture (500 mL) was used to inoculate 5 L of LB broth (supplemented with 2 g/L of L-rhamnose and 100 µg/mL of ampicillin) in a 7 L stirred fermenter (BIOFLO 3000; New Brunswick Scientific, Edison, NJ, USA). The fermenter was equipped with sensors for pH (Metteler-Toledo, MA, USA), dissolved oxygen (Ingold, Leicester, UK) and temperature. Fermentations were carried out as batches at 37°C. Foam was controlled by manual addition of a polypropylene glycol antifoam agent when required. The bacterial growth and nitrilase production were determined in fermentations carried out at various initial pH values (pH 6.0, 6.5, 7.0, 7.5 and 8.0) and at controlled pH values (pH 6.0, 6.5, 7.0 and 7.5). Various agitation speeds (200, 300, 400, 500 rpm) and aeration rates (0.2, 0.4, 0.6 vvm) were tested in different fermentations. In the fermentations carried out at a controlled pH, the pH was controlled by automatic addition of 1N H_2SO_4 and 1N NaOH.

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The fermentations were sampled periodically for measurements of the biomass concentration and the nitrilase activity.

Biomass concentration

The biomass concentration was estimated by measuring the optical density (OD) of the sample at 600 nm using a spectrophotometer (Beckman DU 7400, USA) against a blank of the fresh medium. A standard curve was used to convert the optical density measurements to a dry cell weight (DCW). The standard curve had been made by measuring the optical density of various dilutions of a sample of the broth with a known DCW concentration. The latter had been determined by recovering the cells by centrifugation (10,000 g), washing twice with distilled water, overnight drying (100°C) and weighing.

Nitrilase activity assay

Washed fresh cells (10 mg wet cell paste) were suspended in 2 mL of phosphate buffer (100 mM, pH 7.5) and mandelonitrile (5 mM) was added. The reaction mixture was incubated in a water bath at 37°C for 20 min. The amount of ammonia produced by the enzymatic reaction was estimated by a fluorimetric method [17,22].One unit of nitrilase activity was defined as the amount of enzyme required to produce 1µmol of ammonia per minute under the assay conditions.

Volumetric oxygen transfer coefficient

Volumetric oxygen transfer coefficient (K_La) for the stirred tank bioreactor was determined by the dynamic method [20]. Thus, the air flow to the bioreactor was stopped briefly and the decline in the concentration of dissolved oxygen (DO) because of consumption by the bacterium was recorded. The air flow then resumed at a preset rate and the consequent increase in DO concentration with time was recorded. The oxygen uptake rate Q was calculated as the slope of the DO profile during the period of suspended aeration. The DO concentration profile recorded after the resumption of aeration was used to calculate the K_La [20]; thus:

$$C_L = C^* - \frac{1}{K_L a}\left(\frac{dC_L}{dt} + Q\right)$$

Where C^* is the saturation concentration of dissolved oxygen and C_L is the dissolved oxygen concentration at time any time t. A plot of the measured concentration C_L versus $[(dC_L/dt) + Q]$ gave a straight line with a slope of $1/K_La$.

Results and Discussion

Nitrilase production

Effect of initial and controlled pH: The effect of initial pH in fermentations carried out without subsequent control of pH was examined at a controlled temperature of 37°C. The aeration rate and the agitation speed were held constant at 0.4 vvm and 400 rpm, respectively. In different fermentations, the initial pH values were 6.0, 6.5, 7.0, 7.5 and 8.0. The value of the maximum specific growth rate was highest at 0.17 h^{-1} in the fermentation with an initial pH of 7.0 (Table 1). This initial pH also gave a substantially higher concentration of the biomass compared to the fermentations conducted at higher or lower values of the initial pH.

To better understand the effect of pH on this fermentation, further experiments were carried out at various controlled pH values. The aeration rate, the agitation speed and the fermentation temperature remained fixed at 0.4 vvm, 400 rpm and 37°C, respectively. The results are shown in Table 2. Among the fermentations carried out at various controlled pH values, the fermentation at a controlled pH of 7.0 was clearly the best in terms of the biomass concentration produced and the maximum specific growth rate attained (Table 2); however, the biomass specific nitrilase activity was only about 60% of the value obtained in the fermentation conduced at an uncontrolled initial pH of 7 (Table 1). Therefore, an initial pH of 7 without subsequent control was superior to a pH-controlled fermentation in providing a highly active biocatalyst. In addition, the time to attain the maximum biomass specific enzyme activity was shorter in the fermentation without pH control (data not shown). In production of a recombinant nitrilase of *Alcaligenes* sp. in *E. coli*, [18] also reported a higher biomass specific enzyme activity in cells grown without pH control at an initial pH of 7.

Effect of agitation: Agitation rate is known to affect growth and enzyme production of some nitrilase producing bacteria [23]. Recombinant microorganisms can be particularly sensitive to the agitation regimen in a bioreactor [24]. Therefore, the effect of impeller agitation speed on nitrilase production was examined. The recombinant *E. coli* was grown at different agitation rates (200–500 rpm) and 37°C in different batch fermentations. The aeration rate was always 0.4 vvm. The initial pH was 7.0 and was not controlled. The results are shown in Table 3. The maximum final biomass concentration and the biomass specific nitrilase activity were obtained when the cells were grown at 400 rpm (Table 3). Lower values of agitation intensity adversely affected the final biomass concentration and nitrilase activity possibly because the oxygen transfer rate was reduced by reduced aeration. An agitation intensity of >400 rpm also reduced the final biomass concentration and the enzyme activity (Table 3). This may have been because of the effects of shear stress on the cells [19,24].

In view of the possible effects of oxygen transfer on biomass and enzyme production, the values of the overall volumetric gas–liquid mass transfer coefficient (K_La) were measured at different agitation rates (Table 3). The K_La, or the aeration capacity of the bioreactor,

Initial pH	Maximum biomass concentration (mg/mL)	Maximum nitrilase activity (µmol/min·mg dry cells)	Specific growth rate (h^{-1})
6.0	4.33	0.045	0.07
6.5	4.90	0.051	0.10
7.0	6.92	0.058	0.17
7.5	6.10	0.049	0.10
8.0	5.05	0.047	0.07

[a]The agitation speed and aeration rate were constant at 400 rpm and 0.4 vvm, respectively. All the experiments were carried out in triplicate and the average value was taken.

Table 1: Effect of initial pH on final biomass concentration, the specific growth rate and the final nitrilase activity[a]

pH	Maximum biomass concentration (mg/mL)	Maximum nitrilase activity (µmol/min·mg dry cells)	Specific growth rate (h^{-1})
6.0	3.03	0.040	0.06
6.5	2.99	0.052	0.13
7.0	5.01	0.035	0.19
7.5	3.86	0.045	0.08

[a]The agitation speed and aeration ratewere constant at 400 rpm and 0.4 vvm, respectively. All the experiments were carried out in triplicate and the average value was taken.

Table 2: Effect of controlled pH on final biomass concentration, the specific growth rate and the final nitrilase activity[a]

was the highest at 500 rpm (Table 3) and was progressively reduced by a reduction in the agitation speed (Table 3). Clearly, therefore, oxygen limitation could not explain the reduced growth and nitrilase production seen at the agitation speed of 500 rpm (Table 3). The concentration of extracellular protein and the fermentation pH were not affected by increasing the agitation speed to 500 rpm relative to the results at 400 rpm (data not shown). Therefore, an increased agitation did not physically damage the cells, but affected growth and enzyme production via some of the other shear-dependent mechanisms that have been described [24].

Effect of aeration: In view of the aforementioned effects of oxygen transfer on cell growth and enzyme production, the effect of changes in aeration rate on the fermentation was investigated. The agitation speed was fixed at 400 rpm. The initial pH was set at 7.0 without subsequent control. The other fermentation conditions were as previously specified. Only the aeration rate varied in different batch fermentations. The aeration rate values of 0.2, 0.4 and 0.6 vvm were examined. The data are shown in Table 4. Clearly, the oxygen transfer capacity of the bioreactor (i.e. the K_La) progressively increased with increasing aeration rate (Table 4). The optimal aeration rate was 0.4 vvm. Increasing the aeration rate above 0.4 vvm had a negative effect on both the final biomass concentration and the biomass specific nitrilase activity (Table 4). The most probable reason may be that at higher aeration rate, air flow along the shaft increased and impeller started flooding. An impeller when surrounded by air column is no longer in good contact with liquid and results in meager mixing, reduced air dispersion with less oxygen transfer efficiency. Increased aeration rate seems to be reducing the growth lag as well as time to achieve the maximum growth at a constant agitation rate. The less enzyme activity at higher aeration rate may be due to the oxygen toxicity. Oxygen is vital for the growth of the aerobic organism, however, at higher aeration rate, enzyme activity may be adversely affected. This fermentation therefore required a relatively high oxygen transfer capability in the bioreactor, but not an excessively intense hydrodynamic shear environment [23].

Conclusion

A recombinant *E.coli* was used to produce the nitrilase of *Alcaligenes faecalis* MTCC-126 as an intracellular enzyme. Whole cells of the recombinant bacterium were potently effective as a biocatalyst for the biotransformation of mandelonitrile to mandelic acid (data not shown). In batch fermentation, the optimal conditions for producing a high concentration of the cells with a high biomass specific nitrilase activity were an initial pH of 7.0 without control; a temperature of 37°C; an aeration rate of 0.4 vvm; and an agitation speed of 400 rpm. The recombinant *E. coli* cells biotransformed mandelonitrile to (*R*)-(-)- mandelic acid with remarkable stereoselectivity.

Agitation speed (rpm)	Maximum biomass concentration (mg/mL)	Maximum nitrilase activity (µmol/min·mg dry cells)	K_La (h^{-1})	Specific growth rate (h^{-1})
200	4.02	0.038	58	0.08
300	5.42	0.048	81	0.14
400	6.92	0.057	100	0.17
500	6.31	0.046	120	0.13

[a]The aeration rate remained constant at 0.4 vvm. All the experiments were carried out in triplicate and the average value was taken.

Table 3: Effect of agitation speed on final biomass concentration, the specific growth rate, K_La and the final nitrilase activity[a]

Aeration rate (vvm)	Maximum biomass concentration (mg/mL)	Maximum nitrilase activity (µmol/min·mg dry cells)	K_La (h^{-1})	Specific growth rate (h^{-1})
0.2	5.92	0.051	80	0.13
0.4	6.92	0.058	100	0.17
0.6	6.30	0.050	123	0.11

[a]The agitation speed remained constant at 400 rpm. All the experiments were carried out in triplicate and the average value was taken.

Table 4: Effect of aeration rate on final biomass concentration, the specific growth rate, K_La and the final nitrilase activity[a]

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