Production of a Thermostable Nitrilase in a Lab Scale Stirred Tank Bioreactor

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Abstract

Microbial nitrilases are biocatalysts of industrial interest and being used for the production of amides and acids from their respective nitriles. The production of nitrilases from various sources has been reported with specific catalytic properties at flask level. We attempted the nitrilase production from a Streptomyces sp. MTCC 7546 in a lab scale stirred tank bioreactor under various physiological conditions. The effect of temperature, inducer concentration, agitation, cultivation time and aeration respectively were optimized and it was found that benzonitrile at a concentration of 10 mM, aeration rate in the range of 0.7-1.0 vvm and an agitation of 200 rpm were most suitable conditions for maximum synthesis of intracellular nitrilase. At optimal conditions, the produced dry cell biomass and the productivity of the process were 10.26 g/L and 0.43 g/L/h repectively.

Keywords**:** *Acrylonitrile, Biocatalysis, Bioreactor, Inducer, Nitrilase, Streptomyces*

1. Introduction

The nitrilases are an important class of industrial hydrolytic enzymes belonging to the nitrilase super family and are expressed widely in both prokaryotes and eukaryotes [1]. The majority of the known nitrilases were obtained from bacteria, fungi and plants using a variety of selection methods on media containing nitriles as sole source of carbon and nitrogen or through direct cloning and expression [2, 3]. Nitrile compounds are generally catabolized by microorganisms through direct conversion to carboxylic acids and ammonia using a nitrilase system or it can be metabolized to amide as an intermediate product in presence of nitrile hydratase followed by conversion to corresponding carboxylic acid and ammonia by amidase. Nitrilases have different substrate specificities, making them useful for the hydrolysis of a large number of nitriles. These enzymes are used in preparative organic synthesis utilizing their enantio-, regio- and chemo-selective properties, in bioremediation process (toxic nitrile waste treatment) as well as in degradation of herbicides etc. [4, 5]. Therefore, based on the wide applicability of nitrile hydrolyzing enzymes, they are considered as 'Green Catalyst' for the synthesis of products in high yield with selectivity and without polluting the environment [6, 7].

The thermal stability of nitrilase produced from thermophilic sources is substantially higher than those secreted from mesophilic sources. The maximum activity of nitrilase

produced from *Bacillus pallidus* strain Dac521 and *Acidovorax facilis* strain 72W was observed at 60 ^{o}C and 65 ^{o}C, respectively [8, 9]. Recently, a thermophilic strain of *Bacillus* sp. UG-5B, producing a heat-stable benzonitrilase has been isolated and characterized from polluted industrial waters [10]. Periplasmic nitrilase from *Rhodococcus rhodochrous* was found to be fairly thermostable at temperature of 45 °C [11]. The relatively high stabilities of thermophilic nitrile-metabolizing enzymes, their rapid rates of turn over even at suboptimal temperatures and their broad substrate specificities all contribute to the view that these enzymes may in future be successfully applied to industrial biocatalysis and biotransformation processes [12]. The production of nicotinic acid from a thermostable nitrilase producing *Bacillus* sp. has been studied at various parameters [13]. Though nitrilase posses such unique qualities as well as are beneficial biocatalyst in pharmaceutical and chemical industries, the fermentive approach to produce large amount of nitrilase has been very less explored.

We have previously evaluated the kinetics of enzyme production, immobilization of whole cells by entrapment and also observed the effect of various compounds on the activity of intracellular thermostable nitrilase expressed from *Streptomyces* sp. MTCC 7546 [14]-[16]. The present study was made to investigate the production of nitrilase in lab scale stirred tank bioreactor (7.5 L) under various process parameters.

2. Materials and Methods

2.1. Microorganism and Culture Conditions

The culture was isolated from soils of arid zones of Rajasthan, India using Mineral Base Medium (MBM) supplemented with different mono and di nitriles as inducers (0-20 mM) and the ammonia released during the enzyme action was monitored. The isolate was deposited at Microbial Type Culture Collection (MTCC), Chandigarh, India and characterized as *Streptomyces* sp. MTCC 7546. The isolate was maintained on Luria-Bertani (LB) supplemented with 10 mM adiponitrile at 4 ^{o}C. The organism was cultivated in mineral base medium with slight modification that includes yeast extracts (1 g/l), glycerol (10 g/l), trisodium citrate (0.2 g/l), benzonitrile (10 mM as inducer); 5X mineral base (5 g/l NaCl, 1.35 g/l KH_2PO_4, 1 g/l $MgSO_4$. $7H_2O$, 0.87 g/l K_2HPO_4, 0.05 g/l $CaCl_2$, 1.25 mg /l $FeCl_3$) and trace element solution (0.3 g/l H_3BO_3, 0.2 g/l $CoCl_2$. $6H_2O$, 0.1 g/l $ZnSO_4$. 7 H_2O, 0.03 g/l $MnCl_2$. 4 H_2O, 0.03 g/l $Na_2MoO_4.H_2O$, 0.02 g/l $NiCl_2.6H_2O$, and 0.01 g/l $CuCl_2.2H_2O$). Benzonitrile and trace element solution (1 ml/L) were added into the medium after sterilization.

The liquid media was inoculated from a freshly cultured slant and incubated for 24 h at 45 ^{o}C in an orbital shaker set at 200 rpm. The well-grown cells from the enzyme production medium were harvested by centrifuged at 10,000 rpm for 15 min at 4^{o}C. The cell pellet was washed twice with 0.1 M phosphate buffer (pH 7.4) containing 5 mM EDTA, dissolved in suitable amount of the same buffer and stored at 4 ^{o}C for further use. The cell growth was estimated turbidmetrically at 600 nm. All the experiments were performed in triplicate and mean value was recorded.

2.2. Biocatalytic Assay

Nitrilase activity was assayed at 50 ^{0}C for 15 min in a 1.0 ml reaction mixture that contained 0.1 M potassium phosphate buffer with 5 mM EDTA (pH 7.4), acrylonitrile (10 mM unless stated otherwise), and 100 µl whole cell suspension (cell absorbance 1.0, equivalent to ~ 0.0010 g dry cell weight). The reaction was stopped by addition of 0.05 ml 2N

HCl and centrifuged at 10000 rpm for 10 min at 4^0C. The ammonia released during the incubation was measured by Bertholet reaction [17]. Enzyme activity is expressed in I.U. which is defined as the micromole of ammonia released /min/g of dry cell weight. The optimization of different parameters for the synthesis of enzyme at lab scale stirred tank reactor (Infors AG, Switzerland, 7.5 L capacity, working volume 4.0 L) have been investigated by varying the temperature of incubation for enzyme synthesis, concentration of inducer, time of cultivation, aeration and agitation etc. and finally a comparative evaluation was made for production process under shake flask label and bioreactor label.

3. Results

The nitrilase production from *Streptomyces* sp. and its substrate specificity have shown that the maximum synthesis of enzyme occurs at 45 ^{0}C in presence of various carbon sources and can hydrolyze both mono as well as di-nitrile at a temperature of 50 ^{0}C. The available literatures showed scanty information regarding the mass production of nitrilase. Therefore, we investigated the production of enzyme in a lab scale stirred tank bioreactor at various parameters. The biosynthesis of enzyme and its activity were evaluated at each variable. During the course of studies, we noticed that some of the parameters of the enzyme synthesis in shake flasks varied from production at laboratory scale fermeter. Various parameters such as temperature, inducer concentration and aeration etc. were found to greatly influence the nitrilase production process.

3.1. Effect of Temperature

The effect of temperature on the nitrilase synthesis by *Streptomyces* was examined by carrying out the production at different temperatures (40, 45 and 50 ^{o}C) and the activity along with the cell biomass obtained after 24 h of cultivation are shown in Table 1. At 40 and 50 °C, the growth of the microorganism was less as compared to that of 45 °C. The nitrilase activity of cells grown at 45 ^{o} C was comparatively higher than other temperatures. The results indicated that 45 °C is the optimal temperature for the production of nitrilase in the bioreactor.

3.2. Effect of Cultivation Period

The effect of cultivation period on the growth and nitrilase activity in the bioreactor was examined at different time intervals (18, 24, 30 and 36 h respectively) keeping all other parameters constant and the results obtained are represented in Table 2. It was observed from the table 2 that the growth of organism (dry cell biomass) and nitrilase activity were highest at 24 h of incubation period. At 18 h of production process, nitrilase activity and cell mass were less as compared to that achieved at 24 h. Similarly, the activity of enzyme decreased after 24 h of production but, cell mass was comparatively higher than at 24 h of growth period.

Table 1. Effect of Temperature on the Growth and Nitrilase Activity in Bioreactor

Temperature (°C)	Dry Cell Weight (g/L)	Enzyme activity (I.U.)
40	9.03	18.49
45	10.24	25.15
50	11.66	7.78

Table 2. Effect of Cultivation Period on the Growth and Nitrilase Activity in Bioreactor

Cultivation period (h)	Dry Cell Weight (g/L)	Enzyme activity (I.U.)
18	7.84	10.54
24	10.26	26.19
30	11.82	21.48
36	12.48	17.95

3.3. Effect of Inducer Concentration

Nitrilases are generally known to be inducible in nature and benzonitrile was most suitable inducer for nitrilase production in case of *Sterptomyces* sp. MTCC 7546. We examined the synthesis of nitrilase at different benzonitrile concentrations in the range of 0-20 mM and found that activity of nitrilase was highest at10 mM benzonitrile concentration (Figure 1). At higher or lower concentration of benzonitrile than optimum, there was a decrease in both the cell biomass as well as enzyme activity. The reduced activity at higher concentration may be due to the toxic effect of benzonitrile.

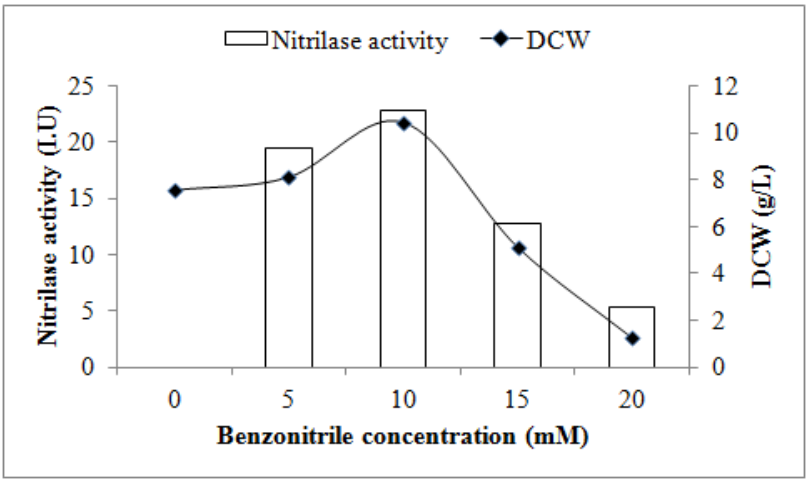


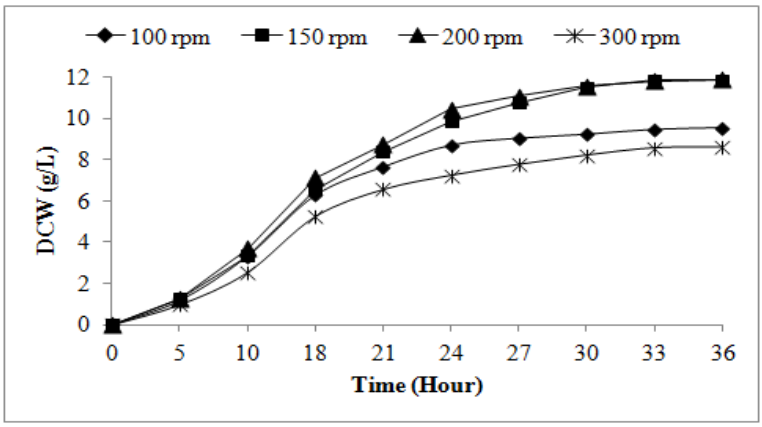
Figure 1. Effect of Benzonitrile Concentration on Nitrilase Production in a Lab Scale Stirred Tank Bioreactor

3.4. Effect of Agitation

Agitation brings about proper mixing of production broth as well as increases the oxygen transfer and thus has a tremendous effect on the productivity of the system. The optimization of agitation for maximum production of any culture needs to carefully monitored, as it has both beneficial as well as deleterious effects (rupture of cells and thus less growth, change in the cell morphology, foam production at high agitation etc). Investigations on the synthesis of nitrilase in the bioreactor were carried out at different agitator speeds (100, 150, 200 and 300 rpm) at optimal conditions and the results are given in Figure 2. At lower speed i.e. 100 rpm, cell biomass as well as enzyme activity was found to be lower than the activity observed at

150 and 200 rpm. The low activity at lower agitation speed could be attributed to the dearth of oxygen being experienced by the organism due to the insufficient mixing. The organism when allowed to grow at higher speed i.e. 300 rpm showed less biomass accumulation and decreased in activity of enzyme. The cells were observed under microscope at the end of the run and the disruption was found to be more as compared to that at lower agitation rate. Although, the organism grew faster at higher agitation, it got sheared more easily which adversely affected the enzyme synthesis. The enzyme activity at 200 rpm was marginally higher and obtained earlier. Hence, an agitation speed of 200 rpm is chosen as the optimal agitation speed for maximum synthesis of enzyme.

A)



B)

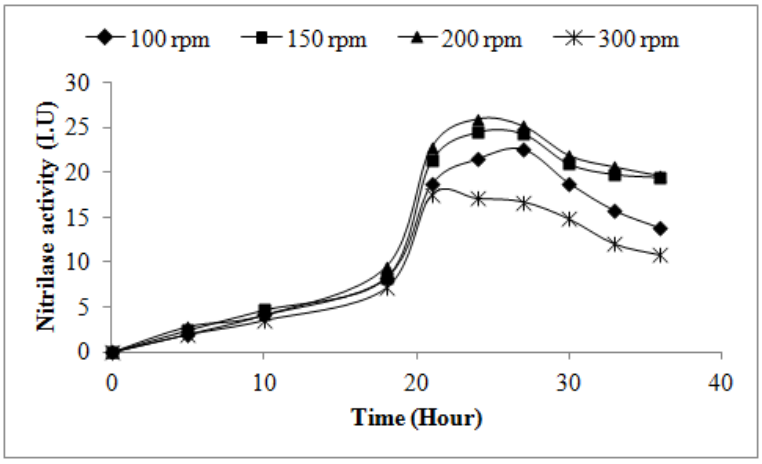


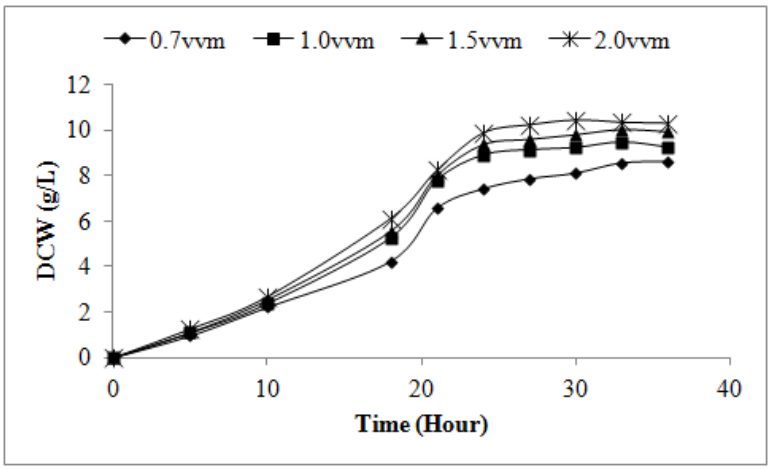
Figure 2. Effect of Agitation on the Growth (A) and Nitrilase Activity (B) in a Lab Scale Stirred Tank Bioreactor

3.5. Effect of aeration

Aeration is essential for maintaining the metabolic activities of aerobic organisms. Aeration also brings about mixing of the bioreactor contents thereby increasing the efficiency of the bioreaction. The dissolved oxygen concentration in a suspension of respiring microorganisms depends on the rate of oxygen transfer from the gas phase to the liquid phase,

rate at which oxygen diffuses to the microorganism and its consumption by the microorganism. During aerobic fermentation, the transfer of oxygen occurs from air bubble into the medium and ultimately to the site of reaction within the cell. Thus, the oxygen transfer from air bubbles, through the liquid medium, to the microbial cells has great importance. More the oxygen transfer more is the oxygen availability for the organism. Oxygen transfer can be increased by increasing the aeration rate. The effect of aeration on the nitrilase production in the bioreactor was evaluated by sparging air to the reactor at different rates (0.7, 1, 1.5 and 2 vvm) and the results on the biomass accumulation and activity are mentioned in Figure 3.

A)



B)

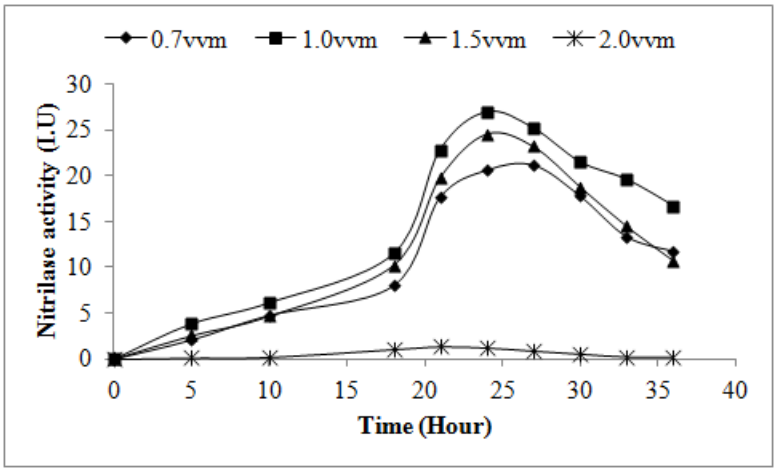


Figure 3. Effect of Aeration on the Growth (A) and Nitrilase Activity (B) in a Lab Scale Stirred Tank Bioreactor

It was observed from the figure 3 that increasing the aeration rate brings about a decline in the enzyme activity of the nitrilase with practically no activity being obtained at higher rates (2 vvm). This may be due to the oxidation of the enzyme protein molecules. At the low aeration rate (0.7 vvm), cell mass and enzyme activity was found to be less than that of 1 vvm. At 1 vvm and 1.5 vvm, the obtained cell biomass was almost similar, but nitrilase

activity was highest for 1 vvm. Higher aeration is beneficial to increase the cell growth; however, it has an adverse effect on the enzyme activity. Therefore, 0.7-1.0 vvm of oxygen transfer rate is chosen as the optimal aeration rate for synthesis of nitrilase in the bioreactor.

Table 3 represents a comparative analysis of different variables for production of nitrilase in shake flask and at bioreactor level using the isolate *Sreptomyces* sp.

Table 3. A Comparative Evaluation of Nitrilase Production in Shake Flask and Bioreactor

Parameters	Shake flask	Bioreactor
Working volume	50 mL	4.0 L
Maximum cell biomass (Xm, g/L)	12.12	10.26
I.U. (µM of NH_4/mL/g dry cell weight	1840	36800
Productivity (Pr, gX/L/h)	0.505	0.428

4. Discussion

Nitrilases are one among various commercially important hydrolytic enzymes. They are used in pharmaceuticals, polymer and other chemical industries to synthesize fine chemicals because of their unique regio-and enatio-selective properties. The enzyme has also been reported in bioremediation process as some of them have broad substrate specificity. Therefore, large scale production of the nitrile hydrolyzing system was needed. There has been very little fermentative approach to produce large scale highly active nitrilase in very short period of time. The first nitrilase mediated bioprocess was performed in 1988 using *R. rhodochorus* J1 for the conversion of 3-cyanopyridine to nicotinic acid but slow conversion rate, low product yield and low substrate tolerance behaved as bottleneck in the nitrilase for large scale production [18]. The successful application of nitrilase in commercial scale requires optimized production technology with low media cost. *Streptomyces* MTCC 7546 was cultured in stirred tank bioreactor and various physico-chemical parameters were studied to produce catalytic active intracellular nitrilase.

The optimal temperature for *Streptomyces* MTCC 7546 in stirred tank reactor for production of active nitrilase was found to be at 45˚C. Till now, most of the nitrile hydrolyzing system has been explored from the mesophillic sources and that to mostly from *Rhodococcus* sps. Very few literatures have discussed about the production of nitrile metabolizing systems from thermophillic sources. One such example is synthesis of nicotinic acid at 60˚C by 3-cynopyrinidase produced from *Bacillus pallidus* strain Dac521 [19]. The nitrilase of *Streptomyces* MTCC 7546 with its thermostable property have larger half life compared to msophilic sources and thus has significant advantage for industrial purpose.

Dissolved oxygen (DO) is one of the most important factors while scaling of aerobic fermentation. DO affects the product formation in many ways; either by influencing metabolic pathway or by changing metabolic fluxes. In aerobic fermentation, the minute change in dissolved oxygen can bring considerable change in physiological alteration in cell metabolism [20]. Agitation and aeration both considerably influence the dissolved oxygen concentration in fermentor. The maximum synthesis of nitrile hydratase using *R. erythropolis* MTCC 1526 in 7-litre reactor achieved at optimum aeration and agitation of 0.5v/v/m and 200 rpm respectively [21]. Attempting the synthesis of nitrilase from *Streptomyces* MTCC 7546, it was found that the aeration has profound effect on the production in 7.5 L lab scale

bioreactor. The optimum aeration for maximum production was recorded in the range of 0.7-1.0 vvm. Similarly, the agitation rate of 200 rpm was most suitable for both enzyme activity and cell growth. Agitation leads to continuous mixing of all the components in the medium as well as the distribution of dissolved oxygen. The high agitation though increases the cell growth but, it enhances shearing of cells which badly affect enzyme synthesis and finally leads to extra formation of foam, whereas low agitation brings improper mixing due to which observed growth was significantly very less. The nitrilase production from *Pseudomonas putida* MTCC 5110 showed that synthesis of cell biomass was highest at 200 rpm, whereas the enzymatic activity was maximum at 150 rpm [22]. Maximum reported nitrilase are inducible in nature and expressed in presence of specific inducer but *Acidovorax facilis* 72W showed no change in activity in presence of different inducers [8]. In case of scale up studies, the effects of varying concentration of inducer on nitrilase production have not been reported. The nitrilase production from *Streptomyces* sp. has shown that benzonitrile in the range of 5-10 mM gave maximum nitrilase synthesis. It was observed that at high concentration of inducer the enzymatic activity decreased drastically. This could be due to cytotoxicity of benzonitrile at higher concentration.

5. Conclusion

A proper large scale production of thermostable nitrilase from *Streptomyces* MTCC 7546 was optimized which can really boost the commercialization of such an economically important biocatalyst. Further optimization regarding the solubility of inducer, distribution of inducer in head space of the bioreactor is required to decrease its toxic effect on the cells. Agitation is playing the crucial role in the dissolution of inducer. An optimum mixing (agitation) of the production medium is must to induce the cells for the synthesis of enzyme. We have assessed the production process for consistency but, the cell biomass produced in fermenter was comparatively lower than that of shake flask study.

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