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Solvent free biocatalytic synthesis of isoniazid from isonicotinamide using whole cell of *Bacillus smithii* strain IITR6b2

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ABSTRACT

A biocatalytic route for the synthesis of isoniazid, an important first-line antitubercular drug, in aqueous system is presented. The reported bioprocess is a greener method, does not involve any hazardous reagent and takes place under mild reaction conditions. Whole cell amidase of *Bacillus smithii* strain IITR6b2 having acyltransferase activity was utilized for its ability to transfer acyl group of isonicotinamide to hydrazine–2HCl in aqueous medium. *B. smithii* strain IITR6b2 possessed 3 folds higher acyltransferase activity as compared to amide hydrolase activity and this ratio was further improved to 4.5 by optimizing concentration of co-substrate hydrazine–2HCl. Various key parameters were optimized and under the optimum reaction conditions of pH (7, phosphate buffer 100 mM), temperature (30 °C), substrate/co-substrate concentration (100/1000 mM) and resting cells concentration (2.0 mg_{dcw}/ml), 90.4% conversion of isonicotinamide to isoniazid was achieved in 60 min. Under these conditions, a fed batch process for production of isoniazid was developed and resulted in the accumulation of 439 mM of isoniazid with 87.8% molar conversion yield and productivity of 6.0 g/h/g_{dcw}. These results demonstrated that enzymatic synthesis of isoniazid using whole cells of *B. smithii* strain IITR6b2 might present an efficient alternative route to the chemical synthesis procedures without the involvement of organic solvent.

1. Introduction

Hydrazides (R-CO—$NHNH_2$), the acylated derivatives of hydrazine are important precursor and intermediates in the synthesis of certain amides, heterocyclic compounds, aldehydes, pharmaceuticals and polymers. These are reported to possess antitumor, antibacterial, anticancer, anticorrosive and anti-inflammatory activities [1–3]. Isonicotinic acid hydrazide, also known as isoniazid (INH) is the first-line antitubercular drug and is given in combination with rifampicin and ethambutol [4,5]. It is a prodrug and must be activated by KatG enzyme of *Mycobacterium tuberculosis*. Isoniazid inhibits the synthesis of mycolic acid, which is an essential component of the mycobacterial cell wall [6]. Chemical methods reported for isoniazid synthesis involves hydrazinolysis of 4-cyanopyridine, isonicotinamide or ethyl isonicotinate with hydrazine hydrate in aqueous or alcoholic solution in presence of sodium hydroxide at 100 °C under reflux conditions. These reactions are spontaneous, exothermic and complete in 4–9 h [7–9]. 4-Cyanopyridine is prepared from expensive γ-picoline whereas less reactive ester requires longer reaction time (from few hours to 2–3 days). Generally these reactions are multistep, expensive, hazardous, energy intensive and can lead to decomposition of desired product.

Yadav et al., [10] explored the possibility of replacing the chemical methods by the enzymatic route, working in non-aqueous media and under milder reaction conditions using lipase as catalyst. This process resulted in 52% conversion in 24 h. They further improved method by using microwave irradiation with immobilized lipase and process led to a conversion of 54% in 4 h [11]. 1,4-Dioxane, solvent used in lipase catalysed synthesis is highly flammable and combines with atmospheric oxygen to form explosive peroxide on prolonged exposure to air. It is a known carcinogen to animals and is classified as a possible carcinogen to human [12–14]. Hence an enzymatic synthesis method with high conversion rate in aqueous medium will provide an economic and greener approach for isoniazid synthesis. Amidases [EC 3.5.1.4] are widely distributed enzymes in nature, with diverse applications [15,16]. The acyltransferase activity of amidase results in transfer of acyl group of amide to co-substrate hydroxylamine or hydrazine in aqueous medium [17–19] and it has been applied for synthesis of few hydroxamic acids [20–25]. Although the ability of amidase to transfer acyl group of amide to hydrazine can be an efficient alternative for biocatalytic synthesis of hydrazides [18,19], surprisingly bioprocess development for hydrazides synthesis using amidase has not been explored till now. To the best of our knowledge, hydrazinolysis of isonicotnamide by amidase has not been applied

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yet for the preparative scale synthesis of isoniazid. The current work focused on the synthesis of isoniazid from isonicotinamide and hydrazine–2HCl in aqueous medium using acyltransferase activity of whole cell amidase of *Bacillus smithii* strain IITR6b2. Whole cells possessed higher acyltransferase to amide hydrolase activity ratio and this ratio was further improved by optimizing hydrazine–2HCl concentration. Various reaction parameters as temperature, pH, substrate/co-substrate concentration, resting cells concentration and time course of reaction were optimized and a fed batch process was developed.

2. Materials and methods

2.1. Chemicals

Isonicotinamide and hydrazine–2HCl were purchased from Sigma Aldrich chemicals Pvt. Ltd. (USA). Isoniazid was obtained from Loba Chemie Pvt. Ltd. (India). The culture media components were obtained from S. D. Fine Chem limited (India). All other chemicals were of analytical or HPLC grade as per requirement, procured from various commercial sources.

2.2. Microorganism

B. smithii strain IITR6b2 was previously isolated from soil sample and preserved in our laboratory. This amidase producing strain with a high amide acyltransferase activity towards heterocyclic amides was used in the present study. Strain was identified by morphological, biochemical tests and 16S rDNA gene sequencing analysis in our previous reported work [26]. The 16S rRNA sequence has been deposited in NCBI GenBank database with accession number JX157878.

2.3. Culture media and growth conditions

B. smithii strain IITR6b2 was cultured in the mineral base medium having following composition (g/l): tri sodium citrate 0.2, glycerol 10, K_2HPO_4 0.87, KH_2PO_4 1.35; 10× mineral base (g/l) (NaCl 10, $CaCl_2$ 0.1, $MgSO_4{\cdot}7H_2O$ 2) and 1 ml/l trace elements solution (g/l) (H_3BO_3 0.3, $Na_2MoO_4{\cdot}H_2O$ 0.03, $NiCl_4{\cdot}6H_2O$ 0.03, $CoCl_2{\cdot}6H_2O$ 0.2, $ZnSO_4{\cdot}7H_2O$ 0.2, $CuCl_2{\cdot}2H_2O$ 0.01 and $MnCl_2{\cdot}4H_2O$ 0.1). 10 mM phenylacetonitrile was added in the sterilized mineral base medium (pH 7) as a sole source of nitrogen. To prepare inocula, the organism was grown aerobically in 50 ml of the sterile medium in a 250 ml Erlenmeyer flask for 36 h at 45 °C and 200 rpm in an incubator shaker. The inocula (0.5% v/v) was added into 100 ml of same medium in a 500 ml Erlenmeyer flask and incubated under similar conditions. The bacterial cells were harvested at mid-exponential phase of growth (OD_{600} = 0.6–0.9) by centrifugation at 10,000 × *g* for 12 min at 4 °C and washed twice with 100 mM phosphate buffer (pH 7). Bacterial cells were suspended in same buffer and referred as resting cells.

2.4. Acyltransferase activity assay

The whole cell acyltransferase activity assay was performed in a reaction mixture (1 ml) having following composition – 400 μl of isonicotinamide solution (250 mM in 100 mM phosphate buffer, pH 7), 500 μl of hydrazine–2HCl solution (1000 mM in distilled water, freshly neutralized with 10.0 N NaOH) and 100 μl of resting cells (1 mg_{dcw}/ml) in phosphate buffer (100 mM, pH 7). Final concentrations of isonicotinamide and hydrazine–2HCl in reaction mixture were 100 and 500 mM respectively. After incubation of reaction mixture at 45 °C for 20 min, the reaction was stopped by adding 10 μl of 2 N HCl and cells were removed by centrifugation at 10,000 × *g* for 8 min. Clear supernatant was collected for HPLC analysis of isoniazid. Reaction mixture without resting cells was also tested for any possible spontaneous chemical synthesis of isoniazid. One unit of acyltranferase activity was defined as that amount of resting cells (mg dry cell = mg_{dcw}) which catalysed the formation of one μmol of isoniazid in 1 min under the assay conditions.

2.5. Amide hydrolase activity assay

The amide hydrolase activity assay was performed in a reaction mixture (1 ml) of the following composition – 800 μl of isonicotinamide solution (125 mM in 50 mM phosphate buffer, pH 7), 100 μl of 50 mM phosphate buffer and 100 μl of resting cell (1.0 mg_{dcw}/ml) in phosphate buffer (50 mM, pH 7). Final concentration of isonicotinamide in reaction mixture was 100 mM. Control reaction was conducted in the absence of enzyme. After incubation of reaction mixture at 45 °C for 20 min, the reaction was terminated by the addition of 10 μl of 2 N HCl and cells were removed by centrifugation at 10,000 × *g* for 8 min. Supernatant was collected for HPLC analysis of isonicotinic acid (INA). One unit of amide hydrolase activity was defined as that amount of resting cells (mg_{dcw}) which catalysed the formation of one μmol of isonicotinic acid in 1 min under the assay conditions.

2.6. Hydrazide hydrolase activity assay

The hydrazide hydrolase activity assay was performed in a reaction mixture (1 ml) of the following composition – 800 μl of isoniazid solution (125 mM in 50 mM phosphate buffer, pH 7), 100 μl of 50 mM phosphate buffer and 100 μl of resting cell (1.0 mg_{dcw}/ml) in phosphate buffer (50 mM, pH 7). Final concentration of isoniazid in reaction mixture was 100 mM. Control reaction was conducted in the absence of enzyme. After incubation of reaction mixture at 45 °C for 20 min, the reaction was terminated by the addition of 10 μl of 2 N HCl and cells were removed by centrifugation at 10,000 × *g* for 8 min. Supernatant was collected for HPLC analysis of isonicotinic acid. One unit of hydrazide hydrolase activity was defined as that amount of resting cells (mg_{dcw}) which catalysed the formation of one μmol of isonicotinic acid in 1 min under the assay conditions.

2.7. Analytical method

HPLC analysis of substrate, product and by product were carried out by Varian Prostar HPLC with waters spherisorb® 10 μm ODS2, 4.6 × 250 mm analytical column. The standards plots of commercially available isoniazid, isonicotinic acid and isonicotinamide were prepared. The analysis was carried out at a flow rate of 1.0 ml/min at 210 nm using 20 mM KH_2PO_4 with 5% (v/v) acetonitrile as mobile phase. 20 μl of the sample was injected.

2.8. Determination of effects of temperature and pH on acyltransferase activity

The effect of temperature on whole cell acyltransferase activity was determined in 1 ml reaction mixture for a temperature range of 25–70 °C under assay conditions (100 mM isonicotinamide, 500 mM hydrazine–2HCl, 50 mM phosphate buffer, pH 7) for 15 min. The optimum pH for the reaction was determined for a pH range of (4.0–10.0) in following buffers (50 mM): sodium acetate buffer (pH 4.0–5.8), potassium phosphate buffer (pH 5.8–8.0), borate buffer (pH 8.0–9.2), and sodium carbonate buffer (pH 9.2–10.0) under standard assay conditions. The effect of molarity of potassium phosphate buffer at optimum pH 7 was studied for a molarity range

of 25–500 mM under assay conditions. Thereafter all further studies were carried out at 100 mM phosphate buffer of pH 7.

2.9. Optimization of substrate and co-substrate concentrations for acyltransferase activity

To study the effect of isonicotinamide concentration on amide acyltransferase activity, varied concentrations of isonicotinamide (10–500 mM) at constant hydrazine–2HCl concentration (500 mM) were used under standard assay conditions (55 °C, phosphate buffer 100 mM, pH 7, 15 min). Effect of hydrazine–2HCl concentration on acyltransferase activity and by product formation was studied in the range of 0–1500 mM keeping isonicotinamide concentration constant (100 mM).

2.10. Effect of reaction temperature on bioconversion

To evaluate the effect of temperature on molar conversion of isonicotinamide to isoniazid and by product formation, enzymatic reactions with optimized substrate/co-substrate concentrations (100/1000 mM) were performed at various temperatures (30, 35, 45 and 55 °C) in 25 ml of 100 mM phosphate buffer (pH 7) using 1.0 mg_{dcw}/ml resting cells. Samples were taken at interval of 15 min for 120 min for HPLC analysis.

2.11. Effect of resting cells on conversion and time course

Cell concentration varying from 1.0 to 2.5 mg_{dcw}/ml were used to find out the optimum concentration of whole cells and time course of bioconversion of isonicotinamide to isoniazid under optimized temperature (30 °C) and substrate/co-substrate (100/1000 mM) concentration in 25 ml of 100 mM phosphate buffer (pH 7).

2.12. Fed batch biotransformation at 50 ml scale

Fed-batch biotransformation was carried out in 250 ml Erlenmeyer flask containing 50 ml of reaction mixture with initial isonicotinamide and hydrazine–2HCl concentration of 100 and 1000 mM respectively in phosphate buffer (100 mM, pH 7) at 30 °C and 2.0 mg_{dcw}/ml resting cells of *B. smithii* strain IITR6b2. Powdered isonicotinamide (0.61 g) and highly concentrated solution (1 ml, 5 M, pH 7) of hydrazine–2HCl were fed in subsequent seven feeds at an interval of 60 min to restrict the residual isonicotinamide and hydrazine–2HCl concentration above 100 and 1000 mM respectively. 500 μl of sample was withdrawn at every 30 min during the reaction and monitored for isoniazid, isonicotinic acid and isonicotinamide concentration. Effort was made to maintain the reaction volume constant around 50 ml. A control experiment was also conducted with same parameters without enzyme for any spontaneous chemical reaction.

2.13. Recovery of isoniazid

A fed batch process was performed at 50 ml scale and after 5 h reaction was stopped by centrifugation at 10,000 × *g* for 20 min at 4 °C. The supernatant obtained was freeze-dried to recover white powder. Isoniazid was further purified by extraction of this powder with 120 ml of methanol for 1 h. The methanolic solution was filtered, evaporated and obtained powder was analysed by HPLC for its purity.

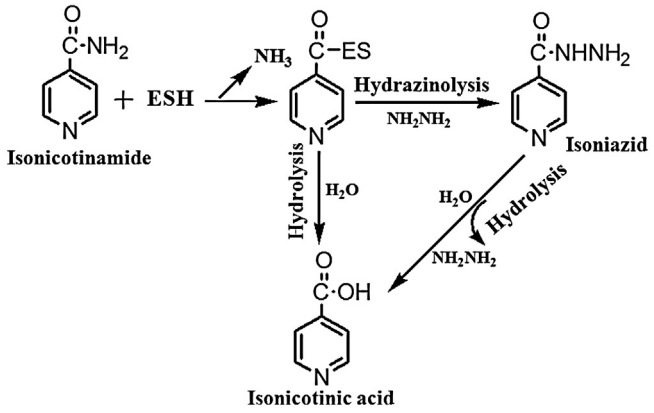

Fig. 1. Enzymatic reactions during *Bacillus smithii* strain IITR6b2 catalysed isoniazid synthesis.

3. Results and discussion

3.1. Enzymatic reactions during B. smithii strain IITR6b2 catalysed isoniazid synthesis

Amidase is capable of catalysing transfer of acyl group of amide, acid and ester to hydroxylamine and water. This transfer of acyl group to hydroxylamine resulted in hydroxamic acid synthesis whereas transfer to water resulted in acid synthesis [17,18,20]. Few amidases have also been reported to catalyse the acyl group transfer from amides to hydrazine, leading to synthesis of acid hydrazides [18,19]. During amidase catalysed hydrazide synthesis an acyl enzyme complex is formed which is subjected to nucleophilic attack by either co-substrate hydrazine or water leading to hydrazide or acid synthesis respectively. [18]. Whole cell of *B. smithii* strain IITR6b2 possessed amide hydrolase (0.87 ± 0.05 U/mg_{dcw}) and amide acyltransferase (2.68 ± 0.24 U/mg_{dcw}) activities but not acid acyltransferase activity. Difference in these two activities might be due to the different affinity of hydrazine and water for acyl enzyme complex and hydrazine being a more potent nucleophile (due to α effect) attacks acyl enzyme complex faster than water. Rate of transfer of acyl group of isonicotinamide to hydrazine (500 mM) was 3 folds higher as compared to amide hydrolase reaction rate. Higher the ratio of acyltranferase to hydrolase activity lesser the by product (isonicotinic acid) will be formed. Thus the synthesis of isonicotinic acid will be limited during the process. One of the objectives of this work was to explore the synthesis of isoniazid by whole cell amidase with minimum formation of by product, isonicotinic acid. Whole cells of *B. smithii* strain IITR6b2 also showed hydrazide hydrolase activity (0.15 ± 0.012 U/mg_{dcw}) when isoniazid was used as substrate in absence of hydrazine–2HCl but it was only 5.6% of amide acyltransferase activity. Kobayashi et al., [19] also reported benzoic acid hydrazide hydrolase activity catalysed by amidase of *Rhodococcus rhodochrous* J1. Based on these activities, the possible reaction mechanism during whole cells catalysed isoniazid synthesis from isonicotinamide is shown in Fig. 1.

3.2. Effect of temperature and pH on acyltransferase activity

To study the influence of reaction temperature on acyltransferase activity the reaction was carried out at various temperature range (25–70 °C). As depicted in Fig. 2 maximum acyltransferase activity was obtained at 55 °C, however no significant increase in activity with temperature was observed at reaction temperature from 45 to 55 °C. Above 55 °C, a sharp decrease in activity was observed due to thermal inactivation of whole cell enzyme. At

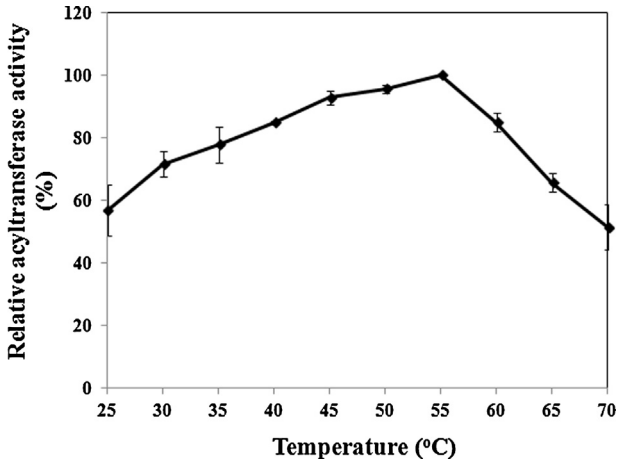

Fig. 2. Effect of temperature on acyltransferase activity. Reaction conditions: isonicotinamide (100 mM), hydrazine–2HCl (500 mM), phosphate buffer (50 mM, pH 7), temperature (25–70 °C), 0.1 mg_{dcw}/ml resting cells. The data are mean ± SD, where $n = 3$.

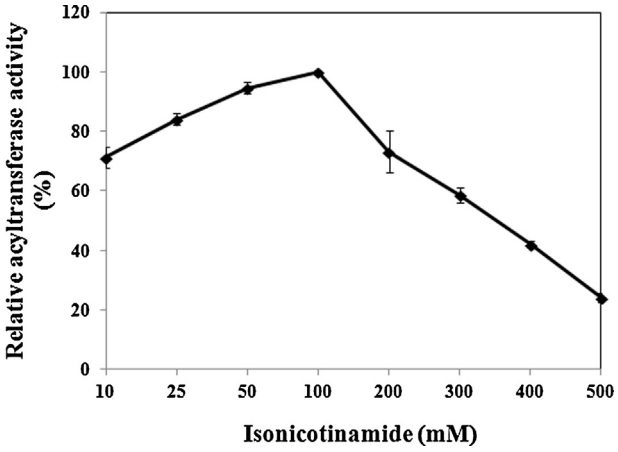

Fig. 4. Effect of substrate (isonicotinamide) concentration on acyltransferase activity. Reaction conditions: isonicotinamide (10–500 mM), hydrazine–2HCl (500 mM), phosphate buffer (100 mM, pH 7), 55 °C, 0.1 mg_{dcw}/ml resting cells. The data are mean ± SD, where $n = 3$.

25 and 70 °C relative acyltransferase activities were 56% and 51% respectively.

The effect of reaction pH on acyltransferase activity was investigated in a pH range of 4–10 using various buffers (50 mM). Results suggested that the whole cell enzyme was active at narrow range and activity increased gradually with increasing pH reaching the maximum at pH 7 (Fig. 3). It was found that it retain 69% and 42% of its maximum activity at pH 6 and 10 respectively. The molarities of potassium phosphate buffer at optimum pH 7 were varied from 25 to 500 mM to find out its optimum value. Potassium phosphate buffer of 100 mM (pH 7) was found to show maximum acyltransferase activity (Supplementary Fig. S1). In earlier studies, the highest acyl transfer activity of amidase of *R. rhodochrous* J1 for benzoic acid hydrazide synthesis was reported at pH 8.5 [19].

3.3. Optimization of substrate and co-substrate concentrations for acyltransferase activity

B. smithii strain IITR6b2 catalysed hydrazinolysis of isonicotinamide which is a bi-substrate reaction involving an acyl group donar (isonicotinamide) and acyl group acceptor (hydrazine–2HCl). It was expected that concentrations of both substrates may influence the acyltransferase activity and side reaction (amide hydrolysis). Thus there was a need to study the effect of substrate/co-substrate concentrations on acyltransferase activity and optimize their concentrations. In first set of experiments, concentration of isonicotinamide was varied from 10-500 mM keeping the concentration of hydrazine–2HCl constant (500 mM). Rise in acyltransferase acivity was observed as the concentration of isonicotinamide increased from 10 to 100 mM with maximum acyltransferase activity achieved at 100 mM of isonicotinamide. Increasing the amide concentration increased acyltransferase activity because of more acyl-enzymes complex formation. A further increase in the amide concentration led to decrease in the enzyme activity and 76% reduction in acyltransferase activity was observed at 500 mM suggesting the inhibition effect of substrate (amide) (Fig. 4). The kinetic constants for isonicotinamide substrate were $K_{m,isonicotinamide} = 4.5$ mM and $V_{max} = 2.87$ μmol min^{-1} $mg^{-1}{}_{dcw}$ of resting cells of *B. smithii* strain IITR6b2.

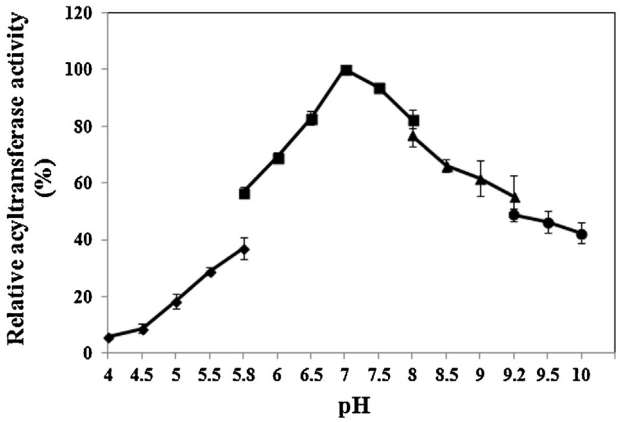

Fig. 3. Effect of pH on acyltransferase activity. Reaction conditions: isonicotinamide (100 mM), hydrazine–2HCl (500 mM), phosphate buffer (50 mM, pH 7), 55 °C, 0.1 mg_{dcw}/ml resting cells. pH profile: acetate buffer (*filled diamonds*), phosphate buffer (*filled squares*), borate buffer (*filled triangles*) and carbonate buffer (*filled circles*). The data are mean ± SD, where $n = 3$.

The effect of hydrazine–2HCl concentration on acyltransferase activity was studied at various hydrazine–2HCl concentrations (0–1500 mM) keeping isonicotinamide concentration (100 mM) constant. It was reported that higher concentration of co-substrate was inhibitory to hydrolysis activity [18]. Hence effect of hydrazine–2HCl concentration on by product formation was also studied. Higher hydrazine–2HCl concentration resulted in increased acyltransferase activity up to 1000 mM and above this concentration decline in activity was observed due to inhibition by hydrazine–2HCl (Fig. 5). The acyltransferase activity was highest at 1000 mM (4.30 ± 0.33 U/mg_{dcw}). The kinetic constant for hydrazine substrate was $K_{m,hydrazine} = 1318$ mM and $V_{max} = 9.8$ μmol $min^{-1}mg^{-1}{}_{dcw}$ of resting cells of *B. smithii* strain IITR6b2.

Higher concentration of co-substrate was found to be inhibitory to hydrolysis of amide and thus by product formation decreased with increasing hydrazine–2HCl concentration (data not shown). At optimum concentrations of isonicotinamide (100 mM) and hydrazine–2HCl (1000 mM), acyltransferase to hydrolase activity ratio further improved from 3 to 4.5 and corresponding reduction in by product formation was 54%. Fournand et al., [18] reported 26% of residual hydrolysis with propionamide substrate at 800 mM hydrazine concentration. This reduction in hydrolysis might be due to the fact that hydrazine being a more potent nucleophile than water had higher affinity for acyl enzyme complex and considerably reduced the undesirable amide hydrolysis. In summary, a high ratio of acyl acceptor (hydrazine–2HCl) to acyl donor (isonicotinamide) improved acyltransferase (hydrolzinolysis) reaction,

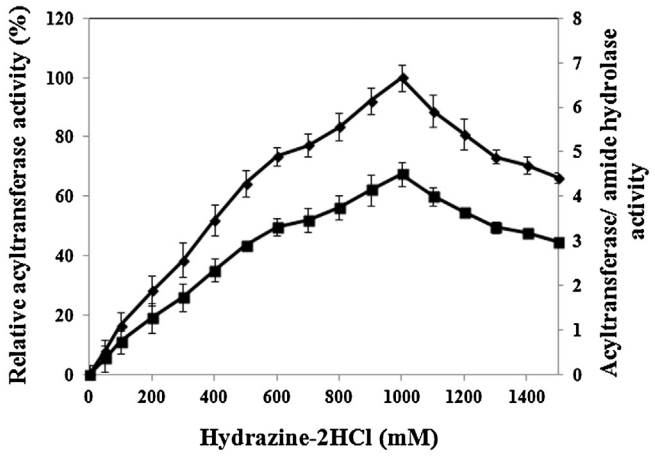

Fig. 5. Effect of co-substrate (hydrazine–2HCl) concentration on acyltransferase activity. Reaction conditions: isonicotinamide (100 mM), hydrazine–2HCl (0-1500 mM), phosphate buffer (100 mM, pH 7), 55 °C, 0.1 mg_{dcw}/ml resting cells. Relative acyltransferase activity (*filled diamonds*) and acyltransferase to amide hydrolase activity ratio (*filled squares*). The data are mean ± SD, where $n = 3$.

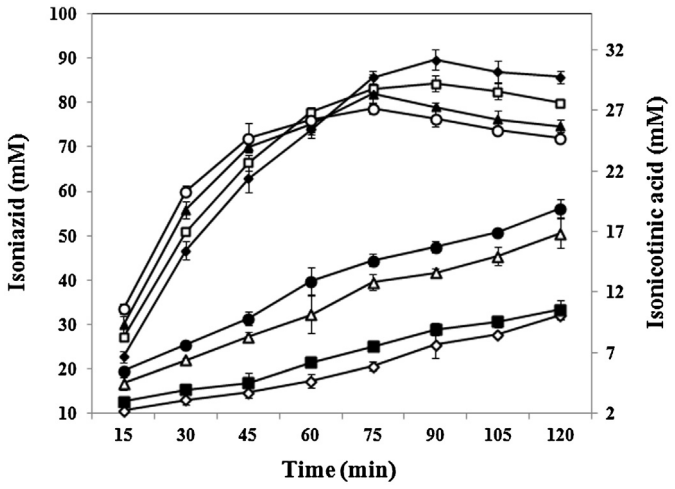

Fig. 6. Effect of reaction temperature on conversion and by product formation. Reaction conditions: isonicotinamide (100 mM), hydrazine–2HCl (1000 mM), 25 ml phosphate buffer (100 mM, pH 7), 1.0 mg_{dcw}/ml resting cells, temperatures (30, 35, 45 and 55 °C). Isoniazid (mM) at 30 °C (*filled diamonds*), 35 °C (*open squares*), 45 °C (*filled triangles*) and 55 °C (*open circles*), isonicotinic acid (mM) at 30 °C (*open diamonds*), 35 °C (*closed squares*), 45 °C (*open triangles*) and 55 °C (*closed circles*). The data are mean ± SD, where $n = 3$.

and suppressed hydrolysis. During the determination the optimum concentrations of isonicotinamide and hydrazine–2HCl, only one substrate concentration was varied whereas the other substrate was kept constant. The possibility of determined concentrations of isonicotinamide and hydrazine–2HCl may be suboptimal cannot be ruled out. Thus to find the true optimum mole ratio of substrates (isonicotinamide and hydrazine–2HCl); reactions were performed with several possible ratios of amide and hydrazine–2HCl. Highest acyltransferase activity was obtained with 100 mM of isonicotinamide and 1000 mM of hydrazine–2HCl (Supplementary Fig. S2). Therefore all the subsequent experiments were performed with 100 and 1000 mM of isonicotinamide and hydrazine–2HCl respectively.

3.4. Effect of reaction temperature on molar conversion and by product formation

It was already found that 55 °C is the optimum temperature for whole cell acyltransferase activity. The objective of this work was to develop a biocatalytic process for optimum synthesis of isoniazid with minimum by product formation. Thus the effects of temperature on molar conversion of isonicotinamide to isoniazid and isonicotinic acid were studied for 120 min at various temperatures (30, 35, 45 and 55 °C). Initial conversion rates were found to increase with an increase in temperature from 30 to 55 °C for first 45 min (Fig. 6). However maximum conversion of 89.6% was achieved at 30 °C in 90 min. There was a marginal difference in the rate of conversion at 45 and 55 °C and maximum conversions achieved were 81.9% and 78.7% respectively in 75 min and thereafter the concentration of isoniazid decreased with time probably due to product hydrolysis. This lower conversion rate at higher temperature can be explained by isonicotinic acid (by product) analysis. By product formation rates were 1.8 and 1.6 folds higher at 55 and 45 °C as compared to 30 °C respectively. It may be due to higher rate of side reactions (amide and hydrazide hydrolysis) at higher temperatures. This study suggested that the final conversion of isonicotinamide to isoniazid was higher at 30 °C with less by product (7.6%) formation. Whole cell amidase of *B. smithii* strain IITR6b2 was reported to be relatively stable at 30 °C with half life of 29 h [26]. As a result the optimal temperature was chosen to be 30 °C for bioprocess development.

3.5. Effect of resting cells on conversion and time course of reaction

The amount of biocatalyst used and time of process is crucial for development of an economic bioprocess. The **e**ffects of whole cell concentration on the molar conversion and time course of reaction were studied in the range of 1.0–2.5 mg_{dcw}/ml under optimized reaction conditions. The concentration of by product (isonicotinic acid) formed was also determined simultaneously. The conversion rate increased with increase in cell concentration and attained a conversion of 90.4% at 2.0 mg_{dcw}/ml while the overall conversion time reduced to 60 min from 90 min (Fig. 7). At higher cell concentration (2.5 mg_{dcw}/ml), however the initial conversion rate was marginally higher but maximum conversion achieved was nearly same and no reduction in reaction time was observed. It indicated that an excess amount of cells would not increase the molar

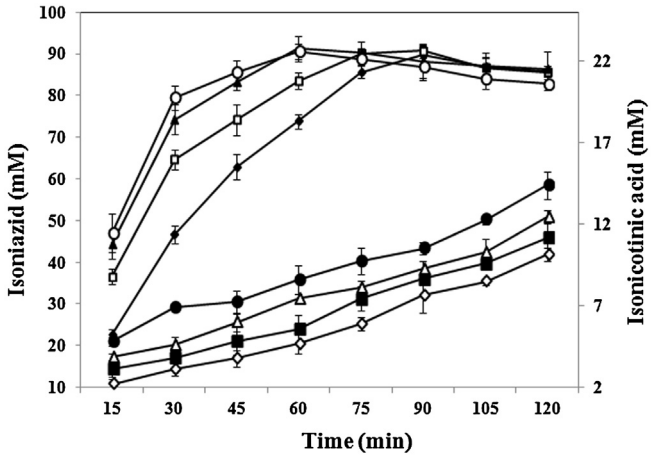

Fig. 7. Effect of resting cells (DCW) on conversion and time course of reaction. Reaction conditions: isonicotinamide (100 mM), hydrazine–2HCl (1000 mM), 25 ml phosphate buffer (100 mM, pH 7), 30 °C. Isoniazid (mM) at 1 mg/ml (*filled diamonds*), 1.5 mg/ml (*open squares*), 2.0 mg/ml (*filled triangles*) and 2.5 mg/ml (*open circles*), isonicotinic acid (mM) at 1 mg/ml (*open diamonds*), 1.5 mg/ml (*closed squares*), 2.0 mg/ml (*open triangles*) and 2.5 mg/ml (*closed circles*). The data are mean ± SD, where $n = 3$.

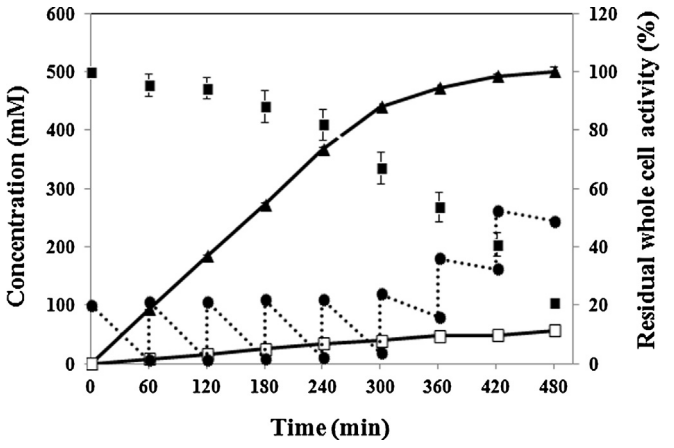

Fig. 8. Time course of production of isoniazid during fed batch biotransformation process. Reaction conditions: 50 ml phosphate buffer (100 mM, pH 7), 30 °C, resting cells concentration (2.0 mg_{dcw}/ml). Isoniazid (mM) (*filled triangles*), isonicotinic acid (mM) (*open squares*) and isonicotinamide (mM) (*filled circles*), residual whole cell activity (%) (*filled squares*). The data are mean ± SD, where $n = 3$.

conversion of isonicotinamide, which might be due to increase in concentration of catalyst above the saturated substrate concentration or mass transfer limitations. A lower amount of the biocatalyst and shorter reaction time will reduce the bioprocess development cost. Hence whole cell concentration of 2.0 mg_{dcw}/ml was used for fed batch process development with intermittent addition of substrates (100/100 mM) at 60 min interval.

3.6. Fed batch bioprocess development at 50 ml scale

Acyltransferase activity of whole cell enzyme was inhibited by isonicotinamide concentration above 100 mM, hence fed batch biotransformation can be a useful strategy in order to obtain high yield of isoniazid. Previous study suggested that under optimized conditions of pH (7, phosphate buffer 100 mM), temperature (30 °C), substrate/co-substrate concentration (100/1000 mM) and cell concentration (2.0 mg_{dcw}/ml), excellent molar bioconversion yield (90.4%) was achieved with in 60 min. Feedings of substrates (100 mM each) were made at an interval of 60 min. The accumulation of isoniazid and isonicotinic acid during biotransformation process with seven feedings of substrates is shown in Fig. 8. There was no inhibitory effect on rate of conversion of isonicotinamide to isoniazid up to third feeding and during 4th feed 79% conversion was observed with respect to conversion achieved in first feed. After 4th feed sharp decline in conversion rate of isonicotinamide was observed and it reduced to 35%, 22.6%, and 8.6% during 5th, 6th and 7th feed. Hence for maximum conversion of substrate to product with minimum by product formation fed batch process should be continued till 4th feed only. A total of 439 mM of isoniazid was accumulated by 87.8% molar conversion of isonicotinamide (500 mM) with only 7.8% by product in 5 h at a production rate of 6.0 g/h/g_{dcw}. During this whole process 32.7% loss in acyltransferase activity and 48.6% decrease in resting cells concentration (measured by optical density at 600 nm) due to lysis was observed after 5 h. Therefore sharp decrease in conversion rate after 4th feed was probably due to combined effects of product inhibition, decrease in acyltransferase activity and cell lysis. This is the first report on biocatalytic synthesis of isoniazid at this conversion rate.

3.7. Recovery of isoniazid

The supernatant obtained was freeze-dried and a white powder (9.1 g) was recovered. On HPLC analysis of this powder it was found that powder contains 33.3% (w/w) isoniazid and 2.5% (w/w) isonicotinic acid. Further purification was done by solvent extraction to recover isoniazid as white powdery solid (3.1 g) with 94% purity.

4. Conclusions

Fed batch bioprocess for isoniazid synthesis has been successfully developed for high molar conversion with limited side reactions using acyltransferase activity of *B. smithii* strain IITR6b2. By optimizing various parameters as hydrazine–2HCl concentration, temperature and resting cells concentration, reaction was directed to the preferred hydrazinolysis hence synthesis of by product (isonicotinic acid) was limited. During this fed batch process after four subsequent feedings a molar conversion (87.8%) of isonicotinamide (500 mM) to isoniazid (439 mM) was achieved in 5 h with only 7.8% by product, isonicotinic acid. Till now the biocatalytic route to isoniazid synthesis using lipase enzyme has been reported, however this process occurs at a slow rate in 1,4-dioxane and resulted in lower conversion (54%). The advantages of present method include aqueous medium, higher conversion in shorter reaction time and mild reaction conditions. These results suggest that *B. smithii* strain IITR6b2 has potential for the commercial production of isoniazid in future. These findings can contribute to industrial production of isoniazid via a biocatalytic route. Further improvements in process can be expected by immobilization of biocatalyst.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.07.010.

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