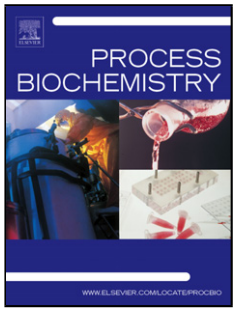

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Thermostable amidase catalysed production of isonicotinic acid from isonicotinamide

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Abstract

The biotransformation of isonicotinamide was investigated using thermophilic intracellular amidase produced from *Geobacillus subterraneus* RL-2a. Various process parameters, including amount of biocatalyst, substrate feeding rate, enzyme-to-substrate ratio and operational thermostability were systematically examined with the aim of achieving complete substrate conversion and high productivity. In 1 litre fed batch reaction containing 0.1 M isonicotinamide, in 0.2 M potassium phosphate buffer (pH 6.5, 200 rpm) and 8 U ml^{-1} amidase activity (12.48 mg dcw ml^{-1}) of whole cells of *Geobacillus subterraneus* RL-2a (as biocatalyst) resulted in a yield of 0.1 M of isonicotinic acid after 50 min reaction time at 70 ºC and a total of 61.55 g isonicotinic acid was produced at a rate of 1.18 g $h^{-1}g^{-1}$dcw respectively. The volumetric productivity was 14.8 g h^{-1} l^{-1}.

Keywords: *Geobacillus subterraneus*; Thermostable; Amidase; Isonicotinamide; Isonicotinic acid; Fed batch

1. Introduction

Amidases are among the most extensively used amide hydrolysing enzymes in industry due to their capacity for the large scale production of optically pure organic acids. In industry these are employed in combination with nitrile hydratase for the production of commercially important organic acids (acrylic acid, p-amino-benzoic acid, pyrazinoic acid, nicotinic acid, hydroxamic etc.) through biotransformation of nitriles [1,2].

Isonicotinic acid or pyridine-4-carbooxylic acid is an attractive compounds due to its pharmaceutical and analytical applications. Isonicotinic acid has been used for the synthesis of inabenfide, a plant growth regulator, isoniazid (antituberculosatic drug), terefenadine, an antihistamine, nialamide, an antidepressant and other pharmaceutically important drugs [3,4,5]. The chemical processes that are used for the manufacturing of isonicotinic acid are hazardous, energy demanding and expensive [6].

The enzyme based processes for the synthesis of important commodity and fine chemicals are gaining importance in the present century. These processes are operable at physiological pH and temperature. The bioprocesses have immense potential in the future to replace the existing chemical processes as the former are highly specific, highly selective, and eco-friendly [7,8].

Isonicotinic acid production from few organisms either with nitrilase activityor nitrile hydratase-amidase activitywas previously reported with *Aspergillus niger* K10, *Fusarium solani* O1, *Nocardia globerula* NHB-2, *Pseudomonas fluorescens* C2 and *Pseudomonas putida* [9,10,5,11,12]. Vejvoda et al. and Malandra et al. explored the potential of fungal nitrilases (*A. niger* K10 and *F. solani* O1) for the bioconversion of 4-cyanopyridine to isonicotinic acid [13,10]. However, the large amount of isonicotinamide (approx. 15% of the total products) was

found as a by-product of the biotransformation of 4-cyanopyridine in addition to isonicotinic acid [14] and 34% of isonicotinamide from purified nitrilase of *A. niger* K10 [15].

In the present work, we focus on the synthesis of isonicotinic acid from isonicotinamide using amidase activity of *G. subterraneus* RL-2a. Different process parameters were optimized for efficient production of isonicotinic acid by fed-batch biotransformation in order to achieve high product concentration.

2. Materials and methods

2.1. Chemicals

Isonicotinamide and isonicotinic acid were purchased from Alfa Aesar, A Johnson Matthey Company (earlier Lanchaster Synthesis). The culture media ingredients were procured from Hi Media, (India). For high pressure liquid chromatography, HPLC grade solvents were from Merck, India.

2.2. Bacterial isolate and cultivation

G. subterraneus RL-2a (MTCC 11502) was isolated from Manikaran thermal spring (Himachal Pradesh, India) and deposited in the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India, and was cultivated as described previously [16].

2.3. Analytical methods

2.3.1. Amidohydrolase (amidase) assay

Amidase activity was assayed using isonicotinamide (50 mM) as substrate in a reaction mixture (2.0 ml) containing 0.2 M potassium phosphate buffer (pH 6.5), 2 mg of resting cells at 70 ºC in a water bath shaker. After 15 min of incubation, the reaction was stopped with equal volume of 0.1 N HCl. The amount of ammonia released in the reaction mixture was colorimetrically estimated using phenate-hypochlorite method [17]. One unit (U) of amidase activity was defined

as that amount of resting cells (mg dry cell = mg dcw) required to release 1 μmol min^{-1} of ammonia by the hydrolysis of amide under assay conditions.

2.3.2. Determination of bioconversion by HPLC

The amount of isonicotinic acid produced in the reaction mixture was determined using an HPLC instrument series 200 Ic pump (Perkin Elmer) equipped with Inertsil® ODS-3 5μm (4.6 x 150 mm) column (GL Sciences, Japan) and 785A Programmable Absorbance Detector (Applied Biosystem). Chromatogram was monitored at 230 nm using mobile phase 0.01 M KH_2PO_4/H_3PO_4 buffer (pH 2.8)/acetonitrile (4:1, v/v) at a flow rate of 1.0 ml per min using NetWin Software (Netel Chromatographs, India). The calibration curves for isonicotinamide (0.1-1.0 mM) and isonicotinic acid (0.1-1.0 mM) were prepared using standards.

2.4 Optimization of reaction conditions of *G. subterraneus* RL-2a amidase for conversion of isonicotinamide to isonicotinic acid.

The optimization of reaction conditions to produce nicotinic acid using amidase of *G.subterraneus* RL-2a showed stability of the enzyme at 70°C in 0.2 M potassium phosphate buffer at pH 6.5 [16]. The other parameters for isonicotinic acid production were optimized in this study.

2.4.1. Time course of enzyme reaction at different substrate concentration

The conversion of isonicotinamide (25-125 mM) to isonicotinic acid with resting cells (3 mg dcw ml^{-1}) was carried out for 2h at 70ºC in 25 ml reaction mixture and samples were withdrawn at every 20 min for estimation of isonicotinic acid and isonicotinamide during the enzyme reaction.

2.4.2. Time course of enzyme reaction at different resting cell concentration

To determine the optimum biocatalyst concentration in reaction required for complete conversion of substrate to product, the reaction was performed using 1.0, 2.0, 3.0, 4.0 and 5.0 U amidases per ml reaction mixture containing 50 mM isonicotinamide at 70 ºC, and after every 10 min, samples were withdrawn for estimation of isonicotinic acid and isonicotinamide.

2.4.3. Time course of enzyme reaction at different temperatures

The operational stability of amidase activity of *G. subterraneus* RL-2a was evaluated by following the time course of enzyme reaction at different temperatures (50, 60, 70 and 80 ºC) by terminating the reaction at different intervals of time viz. 10, 20, 30, 40, 60, 80, 90, 100 and 120 min. Optimized concentrations of substrates and resting cells were used in the reaction mixture.

2.4.4. Time course of isonicotinamide conversion

Substrate concentration and amidase activity were varied as (50 mM: 4.0 U ml^{-1}), (75 mM: 6.0 U ml^{-1}), (100 mM: 8.0 U ml^{-1}), (125 mM: 10.0 U ml^{-1}) and (150 mM: 12.0 U ml^{-1}) in the reaction and samples were withdrawn at every 20 min for 60 min, and analyzed for 100% conversion of substrate to product in shortest possible incubation time and to study the conversion rate with increasing substrate and biocatalyst (resting cell) concentration.

2.4.5. Fed-batch process development at 50-ml scale

In order to scale up the process and to increase the product formation, the reaction was carried out in fed-batch mode in a 250-ml Erlenmeyer flask containing 50 ml of reaction volume containing 100 mM isonicotinamide, respectively, and 8 U ml^{-1} cells in potassium phosphate buffer (200 mM, pH 6.5) at 70 ºC. Using 8 U ml^{-1} amidase activity in the reaction, complete conversion of 100 mM of isonicotinamide to isonicotinic acid was achieved in 50 min. The reaction was performed in a water bath shaker with reciprocal shaking at 70 ºC. Finely powdered isonicotinamide (0.61 g) was added directly to the reaction mixture. A total of 12 feedings (7.32

g isonicotinamide) were added, and a sample (20 µl) was withdrawn for HPLC analysis before every feed for isonicotinic acid.

2.4.6. Fed batch reaction at 1 litre scale

Based on the above experiment, and optimized reaction conditions (in the preceding sections), the reaction volume for conversion of isonicotinamide was scaled up to 1 litre. This reaction was performed in a 1.5 litre fermenter (BioFlow C-32; New Brunswick Scientific, USA). Substrate corresponding to isonicotinamide (12.2 g) was fed every 50 min. A total of 0.5 mol (61.06 g) isonicotinamide was added to the reaction mixture in 250 min. The temperature of the reaction mixture was maintained at 70 ºC, and impeller speed of 200 rpm was set for proper mixing.

3. Results and Discussions

We communicated earlier the bacterial isolation from water samples taken from a thermal spring. Amidase activity profile of *G. subterraneus* RL-2a against various amidases and biotransformation of nicotinamide to nicotinic acid has been published in our pervious article [16]. Since, little attention has been devoted to the production of isonicotinic acid by nitrilases and amidases from thermophilic microorganism. Therefore, here we present our data on the biotransformation of isonicotinamide to isonicotinic acid using the amidase of thermophilic bacterium *G. subterraneus* RL-2a. Although there are a number of available studies on *Geobacillus* amidase, this is the first report on employing *Geobacillus* as a biocatalyst for isonicotinic acid biosynthesis from isonicotinamide [18, 19, 20].

The effects of isonicotinamide concentration ranging from 25 to 125 mM on bioconversion to isonicotinic acid were studied at 70 ºC in 200 mM potassium phosphate buffer (pH 6.5) with a resting cell concentration remained fixed at 3.0 mg dcw ml^{-1}. Full conversion of 50 mM of isonicotinamide was achieved within a time span of 60 min (Fig. 1). It was observed

that increase in concentration of the substrate above 50 mM resulted in slow down of the product formation. This could be due to high isonicotinamide concentration which caused the inhibition. Such inhibition of enzyme activity was observed earlier in the case of amido-hydrolases [16,19,21].

Eventually, full conversion in all case was achieved, though with the increase in substrate concentration it took longer time. For a substrate concentration of 100 mM, full conversion was achieved in 180 min whereas it took 240 min for 125 mM substrate conversion. Consequently, optimal substrate concentration was chosen to be 50 mM.

The amount of resting cells in the reaction mixture was varied from 1.0, 2.0, 3.0, 4.0 and 5.0 U amidases per ml reaction mixture containing 50 mM isonicotinamide at 70 °C (Fig. 2). As the resting cell concentration in the reaction was raised, the rate of formation of nicotinic acid also increased. However, at 5.0 U ml^{-1} resting cell concentration, 49.8 mM, of isonicotinic acid formed in 40 min, which is marginally higher than the 46.7 mM conversion achieved at 4.0 U ml^{-1} in 40 min. This exhibits that additional cells did not improve the conversion significantly. The reaction containing 4.0 U ml^{-1} amidase activity resulted in 100% conversion of the added substrate in 50 min.

In different experiments, the effect of increasing whole cell enzyme concentration with respect to substrate concentration for the conversion of isonicotinamide to isonicotinic acid was studied and shown in Fig. 3. In order to get the best combination of substrate and resting cell in a reaction mixture, their concentration were varied correspondingly from (50 mM: 4.0 U ml^{-1}), (75 mM: 6.0 U ml^{-1}), (100 mM: 8.0 U ml^{-1}), (125 mM: 10.0 U ml^{-1}) and (150 mM: 12.0 U ml^{-1}) The rate of formation of isonicotinic acid was more or less similar in the presence of 50 mM to 100 mM isonicotinamide. The high concentration of substrate (125 mM) and (150 mM) had

inhibitory effect on formation of isonicotinic acid, though the enzyme concentration in the reaction mixture was increased accordingly (10.0 U ml^{-1} and 12.0 U ml^{-1}). The reaction mixture containing 8 Uml^{-1} amidase activity and 100 mM of isonicotinamide showed conversion rate (12.16 gh^{-1}) and at the same time higher yield of isonicotinic acid was also achieved in 50 min. Therefore, 100 mM isonicotinamide and 8.0 U ml^{-1} resting cell concentration was selected further for isonicotinic acid production at 50 ml scale.

It was essential to decide the operating temperature for bioconversion by simultaneously considering the optimum temperature (70 °C) and stability of enzyme. The operational conditions for the biotransformation of isonicotinamide to isonicotinic acid by free cells were investigated in more detail and time course of bioconversions at 50, 60, 70 and 80 °C were investigated for 120 min to choose the optimum temperature of bioprocess for isonicotinic acid synthesis. At 70 °C maximum production of 95.7 mM of isonicotinic acid was achiewed in 50 min (Fig 4). It was observed that when reaction was carried out at low temperature i.e. 50 °C-60 °C conversion rate was slow. 96% and 86% conversion of isonicotinamide to isonicotinic acid was obtained at 70 and 60 °C after 50 min respectively. Formation of isonicotinic acid was more at higher temperatures 80 °C, for the first 10 min (as compared to 70 ºC) but after 20 min, the concentration began to decline due to inactivation of enzyme. Taking into account the enzyme activity and thermostability, the temperature of 70 ◦C was chosen as the reaction temperature in further experiments.One of the factors that restrict the industrial use of biocatalyst is, in general, their relatively low thermostability. Published works rarely reported on nitrilases and amidases fairly stable up to 60 °C but *G. subterraneus* RL-2a has wide range of operational stability and optimum at 70 °C.

The results of feeding of 100 mM substrate in the reaction mixture (50 ml) containing 8 U ml^{-1} amidase are shown in Fig 5. It was observed that until 5 feeds there was no inhibitory effect on the rate of formation of isonicotinic acid, when 100 mM isoniconitnamide was fed at an interval of 50 min. In the 6^{th} feed the substrate hydrolysis rate declined to 95 % producing 568 mMisonicotinic acid.After the 5^{th} feed, the rate of isonicotinic acid formation declined sharply and a total of 484 mM isonicotinamide accumulated in the reaction mixture after 12 feeds. Finally, 716 mM isonicotinic acid was produced in the reaction mixture though a total of 1200 mM isonicotinamide was fed (Fig. 5). The substrate accumulation in the reaction might have affected the amidase activity.

The reaction volume for the conversion of isonicotinamide was scaled up to 1 liter. The reaction mixture (1 liter) contained resting cells (12.48 g dcw) corresponding to 8 U ml^{-1} amidase. A total of 0.5 mole of isonicotinamide fed in 5 feeding of 0.1 mol (every 50 min) was converted to isonicotinic acid in 4 h 10 min. This process resulted in formation of isonicotinic acid 61.55 g at a rate of 1.18 g h^{-1} g dcw^{-1} catalytic productivity. The time and space productivity was 14.8 g h^{-1} l^{-1} in reaction containing 8 U ml^{-1} enzyme.

The available reports show scarce information regarding the enzymatic process of isonicotinic acid production from isonicotinamide. Therefore, comparison with process developed for synthesis of isonicotinic acid from 4-cyanopyridine, would explain the advantage for current process. The aromatic nitrilases from *A. niger* and *F. solani* showed very high specific activities for 4-cyanopyridine [15, 9, 13]. However, a drawback of this procedure was the production of isonicotinamide (about one-third of the total product) as a by-product. Later, Vejvoda et al. used a cascade of immobilized fungal nitrilase and bacterial amidase on 1 ml Butyl Sepharose column and feeding of 40 mM 4-cyanopyridine producing 3.10 g isonicotinic

(99.8% purity) in 35 h with time and space productivity of 94 mg l^{-1} h^{-1} [22]. In continuous stirred membrane reactors (CSMRs) cascade loaded with nitrilase (6.5 U) and amidase (5 U) as cell-free extracts immobilized in cross-linked enzyme aggregates (CLEAs) produced 3.36 g isonicotinic acid (99.9%) in 52 h with the time and space productivity of 118 mg l^{-1} h^{-1} when 4-cyanopyridine was pumped at the rate of 10.5 ml h^{-1} [10].

Vejvoda et al. showed interest in bioproduction of isonicotinic acid when they found remarkable hydrolytic activity for isonicotinamide from *R. erythropolis* A4 [22]. Substrate inhibition is a common problem in enzyme-mediated processes that can be partially overcome by the fed-batch method [5].

Biotransformation process is superior to the previously described processes by Vejvoda et al. and Malandra et al. which involved use of immobilization of fungal nitrilase and bacterial amidase [13,10]. *G. subterraneus* RL-2a amidase achieved the time and space productivity of 14.8 g h^{-1} l^{-1} nicotinic acid which is much higher than the above processes.

Conclusions

This is the first report that describes the process development for the synthesis of isonicotinic acid from isonicotinamide using amidase activity of *G. subterraneus* RL-2a MTCC 11502. The enzyme from *G. subterraneus* RL-2a exhibited favourable biocatalytic properties-good operational thermostability and ability to achieve full conversion relatively rapidly. To the best of our knowledge, In terms of time and space, the production of isonicotinic acid (14.8 g $h^{-1}l^{-1}$) from their corresponding amides in this study is highestas compared to amidase systems reported hitherto. Further, subsequent gene expression and protein engineering of this amidase could further improve its catalytic efficiency towards amides.

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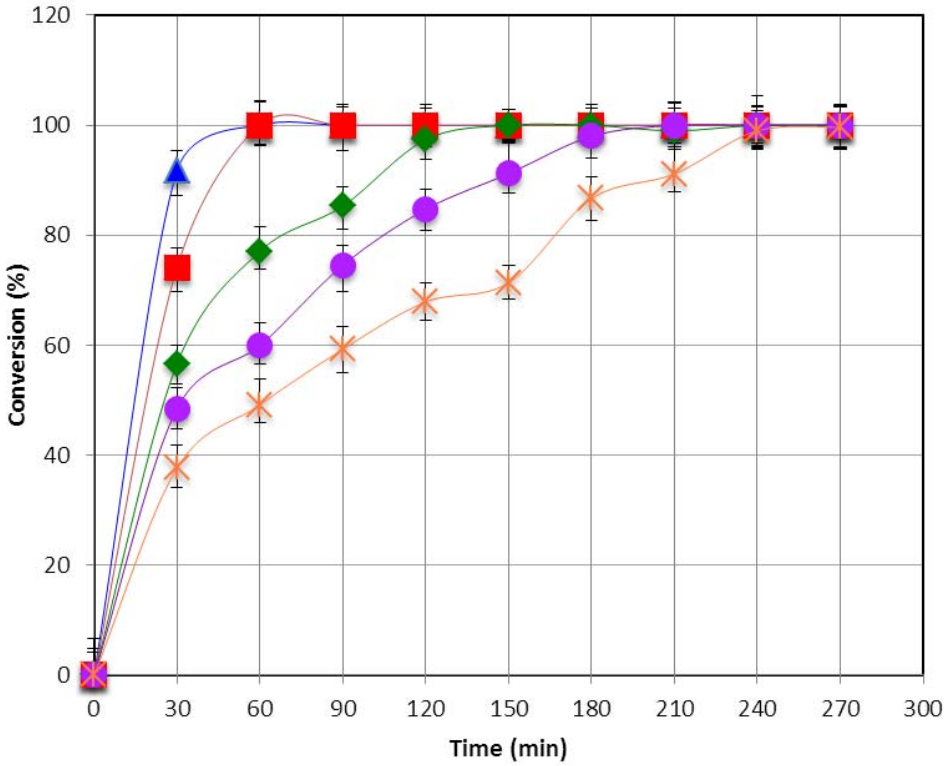
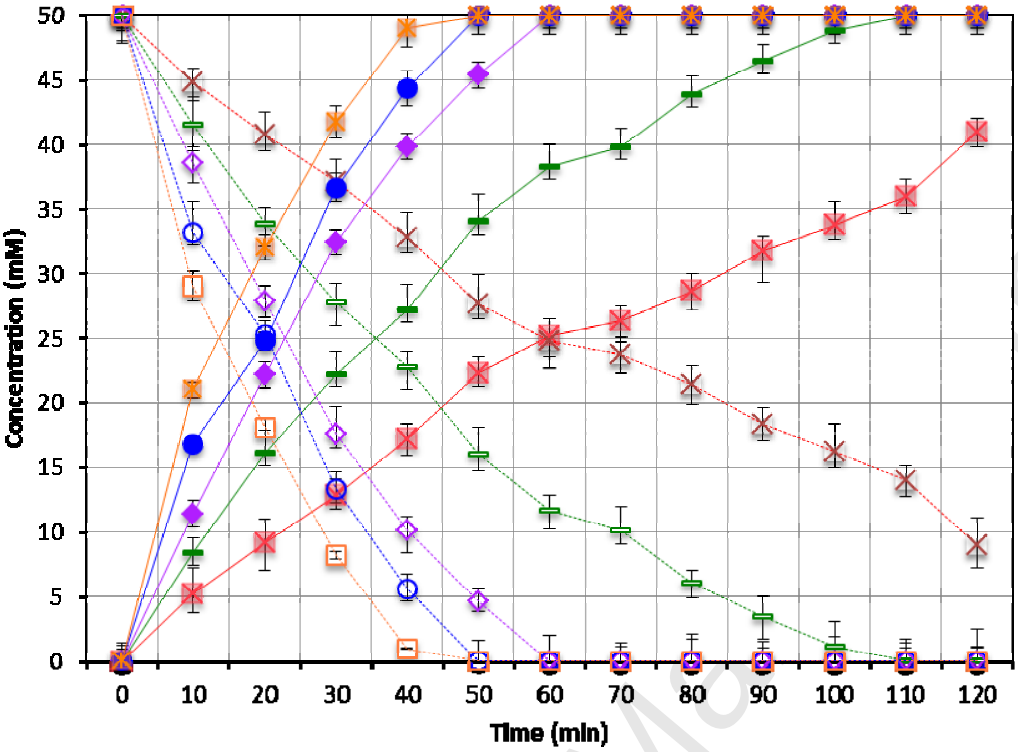


Fig. 1.



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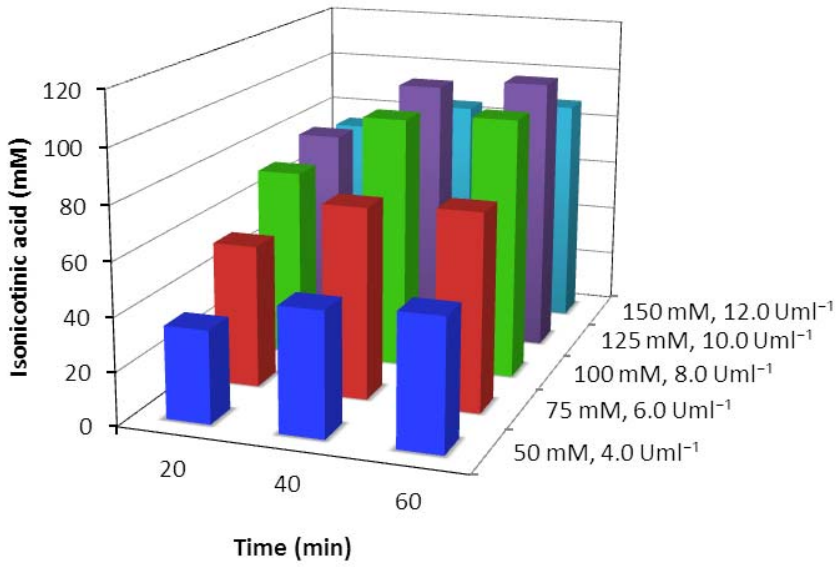

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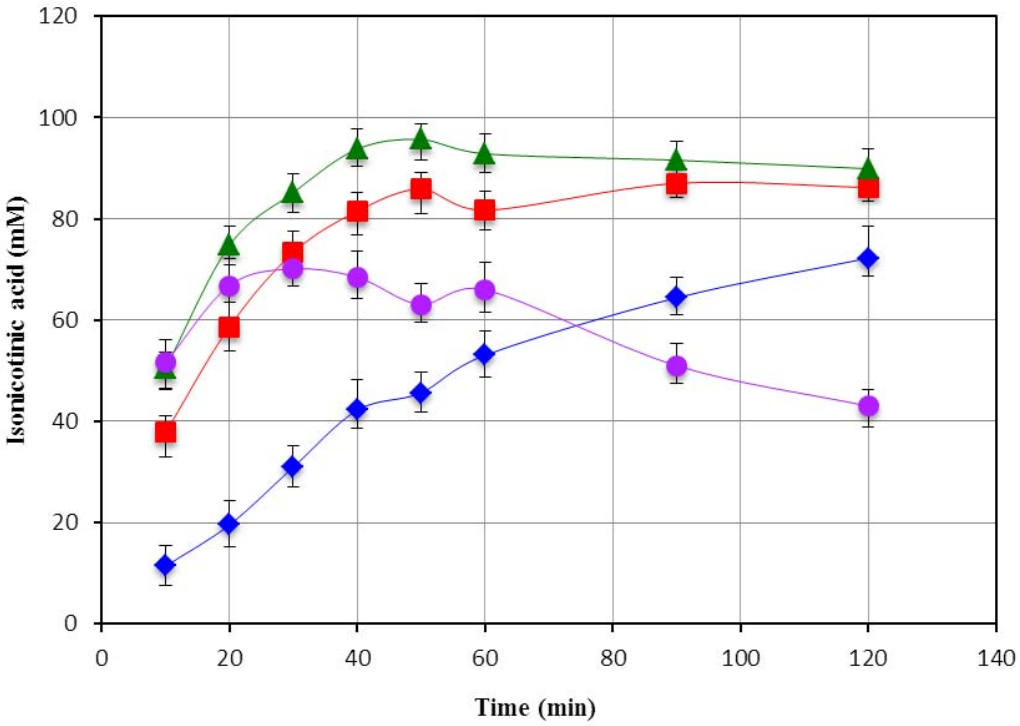

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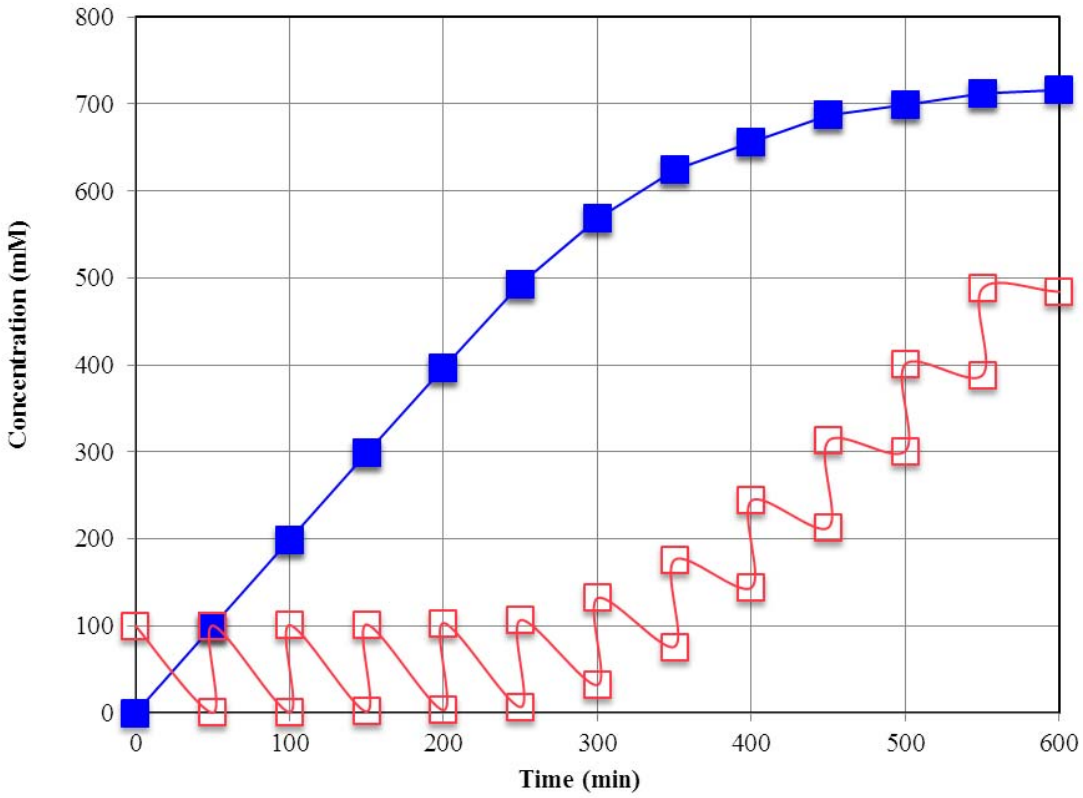

Fig. 5.

Figure captions

Fig. 1. Effect of initial substrate concentration on the conversion catalyzed by resting cells of *G. subterraneus* RL-2a. Reactions were carried out at 70°C with different initial isonicotinamide concentration: 25 mM (-▲-), 50 mM (-■-), 75 mM (-♦-), 100 mM (-●-) and 125 mM (-×-) in 0.2 M potassium phosphate buffer (pH 6.5), containing resting cells (3 mg dcw ml^{-1} fresh cell weight). All experimental data are means ± standard deviation (SD) of triplicate determinations.

Fig. 2. Effect of resting cells concentration on the formation of isonicotinic acid in the reaction containing 100 mM isonicotinamide. Reactions were carried out at 70°C in 0.2 M potassium phosphate buffer (pH 6.5) with specified cell concentrations in the reactiom mixture as per legend. (solid symbols isonicotinic acid, empty symbols isonicotinamide) (■) 1.0 Uml^{-1}; (□) 1.0 Uml^{-1}, (▬) 2.0 Uml^{-1}; (▭) 2.0 Uml^{-1}, (♦) 3.0 Uml^{-1}; (◊) 3.0 Uml^{-1}, (●) 4.0 Uml^{-1}; (○) 4.0 Uml^{-1}; (▲) 5.0 Uml^{-1}; (Δ) 5.0 Uml^{-1}. All experimental data are means ± SD of triplicate determinations.

Fig. 3. Effect of *G. subterraneus* RL-2a resting cells and isonicotinamide concentration on the production of isonicotinic acid. The hydrolytic reactions were carried out at 70ºC in 0.2 M potassium phosphate buffer, pH 6.5 with with specified cell concentrations and substrate concentrations in the reaction mixture as per legend. All measurements were done in triplicates.

Fig. 4. Time course of amidase catalysed production of isonicotinic acid at various temperatures using resting cells (6.24 mg dcw ml^{-1} fresh cell weight) of *G. subterraneus* RL-2a (-▲-, -■-, -♦-, -●- indicate the reaction at 50, 60, 70 and 80°C respectively). Data represent means ± SD of three independent experiments.

412 **Fig. 5.** Fed-batch reaction profile of isonicotinamide bioconversion by *G .subterraneus* RL-2a
413 amidase in 0.2 M potassium phosphate buffer, pH 6.5 at 70ºC. Accumulation of isonicotinic acid
414 and isonicotinamide in 50 ml reaction mixture containing resting cells corresponding to 8 U ml^{-1}
415 amidase activity. Status of isonicotinamide and isonicotinic acid concentration in reaction has
416 been denoted by (□) and (■) respectively. Upper and lower (□) denote the isonicotinamide
417 concentrations after and before the addition of 0.1 M isonicotinamide per 50 min respectively.
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420