Bio-statistical enhancement of acyl transfer activity of amidase for biotransformation of N-substituted aromatic amides

(Received June 21, 2015; Accepted December 18, 2015)

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Acyl transfer activity (ATA) of amidase transfers an acyl group of different amides to hydroxylamine to form the corresponding hydroxamic acid. Bacterial isolate BR-1 was isolated from cyanogenic plant Cirsium vulgare rhizosphere and identified as Pseudomonas putida BR-1 by 16S rDNA sequencing. This organism exhibited high ATA for the biotransformation of N-substituted aromatic amide to the corresponding hydroxamic acid. Optimization of media, tryptone (0.6%), inducer, pH 8.5, and a growth temperature 25°C for 56 h, resulted in a 7-fold increase in ATA. Further, Response Surface Methodology (RSM) and multiple feeding approach (20 mM after 14 h) of inducer led to a 29% enhancement of ATA from this organism. The half life (t1/2) of this enzyme at 50°C and 60°C was 3 h and 1 h, respectively. The ATA of amidase of Pseudomonas putida BR-1 makes it a potential candidate for the production of a variety of N-substituted aromatic hydroxamic acid.

Key Words: cyanogenic plants; hydroxamic acid; nicotinamide; rhizosphere

Introduction

The plant microbe interactions in the rhizosphere affect plant health, growth and productivity. The chemicals secreted by plant roots immensely influence the microbial diversity of the rhizosphere. These microbes have been isolated and reported as plant growth-promoting rhizobacteria (PGPR) (Zahir et al., 2001). PGPR produce extracellular growth-promoting chemical substances, phytohormones, iron chelating hydroxamate siderophores, antibiotics and HCN. These improve plant growth by reducing the population of major root pathogens, inducing plant resistance, mineralizing soil nutrients, and also transporting, iron from soil to roots thereby making Fe3+ less available for certain native microflora (Patten and Glick, 2002). Enzymes involved in the production of hydroxamate types of siderophores by PGPR are not so well documented (Schrettl and Haas, 2011). It can be implicated that the ATA of amidase in microorganisms help in the transfer of an acyl group from amides to hydroxylamine (an intermediate compound in the nitrogen cycle) for the synthesis of the respective hydroxamic acid, as well as hydroxamate siderophores. A number of cyanide/nitrile/amide degrading microorganisms with amidase activity have been reported from soil (Chacko et al., 2009). However, much less information is available on amide transformation into hydroxamate siderophores by microbes of the rhizosphere of cyanogenic plants. Therefore, it can be expected that microorganisms in the rhizosphere of cyanogenic plants biotransform different cyanide/nitrile/amide compounds into the corresponding carboxylic acids, hydroxamic acid and hydroxamate siderophores by the pathways shown in Fig. 1. Most of the earlier studies were confined mainly to the production of aliphatic or aromatic hydroxamic acid production (Bhatia et al., 2012; Fournand et al., 1998). These hydroxamic acids form stable chelates with metal ions and are constituents of several interesting compounds, such as: growth factors, food additives, antibiotics, tumour inhibitors, siderophores and enzyme inhibitors, which have tremendous applications in biology and medicine (Bertin et al., 2003; Rao et al., 2010). Therefore, it will be worthwhile to focus on such microbes which can be used for the production of N-substituted aromatic hydroxamic acids (nicotinyl hydroxamic acid) as later is considered to be safer for in-vivo medical applications. Bearing the above facts in mind, nitrile/amide
hydrolyzing microbes has been isolated from the rhizosphere of thirteen cyanogenic plants. Here, we report on the screening, selection, identification and optimization of the culture conditions of a high ATA-producing isolate BR-1 for the synthesis of nicotinyl hydroxamic acid.

Material and Methods

Chemicals. All the chemicals used in this study were of analytical grade and purchased from renowned commercial manufacturers such as Lancaster Synthesis, England, Merck India, Sigma Aldrich USA, and Hi Media, (Mumbai, India).

Isolation of amide-degrading bacteria from the rhizosphere of cyanogenic plants. Thirteen cyanogenic plants were selected from different regions of Himachal Pradesh, India. Rhizospheric soil of these cyanogenic plants was collected in sterile tubes, brought to the laboratory and stored at 4°C until further use. Further, isolation of amide-degrading bacteria was carried out using the method given by Bhatia et al. (2013a).

Assay method for screening of bacterial isolates for ATA. The resting cells of different isolates were screened for the production of ATA of amidase using the Brammar and Clarke (1964) method. The test reaction contained two substrates, viz. nicotinamide (100 mM) and hydroxylamine (10 mM) and methanol in the ratio of 9:1 in HPLC grade water and pH of the mobile phase adjusted to 3.5 with ortho-phosphoric acid.

Phenotypic and biochemical characterization. Gram (positive/negative) staining was carried out by using HiMedia Gram staining kit according to the manufacturer’s instructions. The bacterial isolate BR-1 was characterized up to genus level by biochemical tests at the Institute of Microbial Technology, Chandigarh, India.

Molecular characterization. The identity of this organism was further confirmed by the 16S rDNA gene sequencing method. DNA was extracted and amplified by a polymerase chain reaction (PCR) using the following universal 16S rDNA gene primers, 8F: 5'-AGAGTTTGATCCTGCTCA-3' and 1492R: 5'-TACG GYTACCTTG TTA CGACTT-3'. PCR was conducted by subjecting a reaction mixture to initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, 55°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min. The amplified product was sequenced at Xcelris Labs Ltd. Gujrat (India).

16S rDNA sequencing and BLAST analysis of the amplified gene. The 16S rDNA gene sequence was compared using the BLAST algorithm at the NCBI. A phylogenetic tree was constructed using the neighbor-joining method of Clustal W (Anisimova and Gascuel, 2006; Castresana, 2000).

Optimization of biotransformation conditions for the synthesis of N-substituted aromatic hydroxamic acid. A one variable at a time (OVAT) approach was used for the optimization of culture conditions for the maximum production of ATA. A number of variables such as media (M1–M18), nitrogen source, inoculum size (1–10%), incubation time (0–10 h), pH (4–13), temperature (5–70°C), 10 mM inducer (different types of amides and nitriles), inducer concentration (0.1–1.0%), multiple feeding of inducer and thermal stability were taken into consideration for assessing their effect on the production of ATA.

Plackett-Burman design. Ten variables, tryptone (0.6%), yeast extract (0.1 gL−1), Na2HPO4 (2.5 gL−1), KH2PO4 (2.0 gL−1), MgSO4·7H2O (0.5 gL−1), FeSO4·7H2O (0.03 gL−1), CaCl2·2H2O (0.6 gL−1), inoculum size (5%), inducer isobutyronitrile (0.3%) and pH (7) at two levels, minimum and maximum, were used. These variables were investigated by 12 experiments using Design Expert (Version 8.0.7.1) (Plackett and Burman, 1946). The effect of each variable was calculated using the following equation:

$$E = \frac{\sum M + \sum M - }{N},$$

where $E$ is the effect of the tested variable, and $M+$ and $M−$ are the responses (ATA U/mg dcm) of trials at which the parameter was at its higher and lower level, respectively, and $N$ is the number of experiments carried out.

Central composite design (CCD). The concentration of different independent variables which showed a positive
Validation of the statistical model. The statistical model was validated for the production of ATA by performing experiment at shake flask under a predicted set of conditions.

Multiple feeding of the inducer for hyper induction of the ATA of amidase. *Pseudomonas putida* BR-1 is a slow-growing bacterium which produces a maximum ATA in its exponential phase, i.e. 56 h. The culture of *Pseudomonas putida* BR-1 was fed with isobutyronitrile into 4 different levels in five different sets each with a constant level, a high exponential level, a low exponential level, and a decreasing level, at intervals of 0, 14 h, 28 h, and 42 h. Cells were harvested after 56 h, and growth was determined turbidometrically at 600 nm and the ATA was measured as discussed previously.

Stability. The thermal stability of amidase of *Pseudomonas putida* BR-1 for ATA was studied by preincubated resting cells of this organism at different temperatures (4°C, 15–60°C) and the ATA was assayed at intervals of one hour.

Substrate affinity. The ATA of amidase of *Pseudomonas putida* BR-1 was determined for a variety of amides. A solution of 50 mM of different amides was prepared in their respective solvents. All amides were prepared in deionized water, except for L-alaninamide hydrochloride, D,L-phenylalaninamide, phenylmalonamide (prepared in 1 M NaOH), benzamide, and hexanoamide (prepared in 50% (v/v) ethanol). The ATA for each amide was analyzed as discussed earlier in the Assay Method section.

Results

Isolation and screening

Two hundred and fifty-six isolates of bacteria were isolated by an enrichment technique from the rhizosphere of thirteen cyanogenic plants present in Himachal Pradesh, India. Out of these, seventy-eight isolates showed activity for different amides, as well as for nitriles, and thirty-eight isolates were found positive for the degradation of *N*-substituted aromatic amides only. One isolate BR-1, from the rhizosphere of cyanogenic plant *Cirsium vulgare* produced a denser magenta colour, indicating a higher ATA of amidase. The results showed that the isolate BR-1 was a hyper producer of ATA (6.29 ± 0.01 Umgdcm⁻¹) for *N*-substituted aromatic amide and also indicated that amide-degrading hydrolytic bacteria are widespread in the rhizosphere of cyanogenic plants.

Identification of isolate BR-1

Morphological and physiological characterization. Isolate BR-1 is Gram negative, aerobic and rod shaped having cream color colonies, entire margin and flat elevation. This isolate was oxidase and catalase positive and fermented dextrose, which indicated that the isolate BR-1 belongs to the genus *Pseudomonas*.

16S rDNA sequencing. The identity of the isolate BR-1 was further confirmed up to sp. level by 16S rDNA sequencing. An approximately 1337 bp 16S rDNA sequence was obtained from the isolate BR-1 which was aligned with 16S rDNA sequences available in the GenBank database of NCBI. A phylogenetic tree was constructed using the neighbor-joining method from 16S rDNA sequences of different *Pseudomonas* species. The phylogenetic analysis indicated that the 16S rDNA sequence of the isolate BR-1 was highly homologous (99%) with the sequence of *Pseudomonas putida* strain CY04 (NCBI accession No. JX082200.1), *Pseudomonas putida* strain Zn-2 (NCBI accession No. JX441333.1) and *Pseudomonas putida* strain jvu23 (NCBI accession No. JQ701740.1) (Fig. 2). The 16S rDNA sequence of the isolate BR-1 was deposited in GenBank under accession number JX649916. Based on these results, isolate BR-1 was identified as *Pseudomonas putida* BR-1.

Optimization of biotransformation conditions for the synthesis of *N*-substituted aromatic hydroxamic acid

Media, carbon and nitrogen source. *Pseudomonas putida* BR-1 was cultured in eighteen different media (M1–M18), of which seven media were a carbon-rich source and the remaining eleven were mineral salt media. It is evident that in mineral media, this bacterium has less growth compared with the carbon-rich media, but produced a fairly good ATA. It seems that salts in the medium M5 favored a higher production of ATA (10.11 ± 0.01 Umgdcm⁻¹). In medium M5, tryptone works as an excellent source of carbon as well as nitrogen, with (0.2%) tryptone proving to
be the best source, giving a high ATA (15.24 ± 0.05 U mgdcm⁻¹) vis-à-vis a cell mass of 2.6 mg ml⁻¹.

**Inducer.** *Pseudomonas putida* BR-1 was grown in the production medium (M5) containing different aliphatic, aromatic and heterocyclic nitriles, and amides, which were tested for their ability to induce the production of the ATA of amidase. Isobutyronitrile was found to be the most suitable inducer with a reasonably higher production of biomass (3.2 mgdcm⁻¹) and ATA (17.14 ± 0.01 U mgdcm⁻¹) (Fig. 3). Isobutyronitrile (0.3% v/v) in the medium resulted in hyper induction of the ATA i.e. 18.17 ± 0.02 U mgdcm⁻¹ and at concentrations less/higher than 0.3% of isobutyronitrile, a decrease in ATA was observed.

**Inoculum size and the time course of incubation.** A six-fold enhancement of ATA (39.69 ± 0.04 U mgdcm⁻¹) was observed using 5% (v/v) inoculum. The decrease in the enzyme activity with the increase in the inoculum size beyond 5% may be due to the faster depletion of nutrients in the medium as a very high number of cells were present in the cultures from the very beginning. The production of the ATA reached its maximum (58.77 ± 0.03 U mgdcm⁻¹) with a biomass of 5.4 mg ml⁻¹ in the exponential phase of growth, i.e. 56 h of incubation, and, after that, a decline in enzyme production was observed while OD at 600 nm of the culture continued to increase.

The effect of pH and temperature.** The most favorable pH for the production of ATA was 8.5. The low pH (5.5–6.5) of the medium had a negative effect on the production of ATA of this organism. At pH 8.5, the growth of *Pseudomonas putida* BR-1 was slightly less than pH 7.0, but it produced a higher ATA, i.e. (63.65 ± 0.02 U mgdcm⁻¹). The growth, as well as the ATA, of *Pseudomonas putida* BR-1 increased with an increase in temperature and reached its maximum at 25°C (71.12 ± 0.03 U mgdcm⁻¹). Beyond this, this organism did not grow well and yielded less biomass with very little ATA. This bacterium did not grow above 40°C and the production of ATA also declined.

The combined effect of different medium components using Plackett-Burman design.** A set of twelve experiments exhibited a variation in ATA ranging from 34.07 ± 0.03 to 77.71 ± 0.02 U mgdcm⁻¹ as shown in Table 1. The results obtained by performing experiments with a Plackett-Burman design using ten variables were further used to construct a Pareto chart (Fig. 4) to determine the significance of different variables on the production of ATA.

**Regression analysis** was performed on results obtained by Plackett-Burman design and a first-order polynomial equation was derived to explain the effect of the various independent variables on enzyme production:

\[ \text{ATA (U mgdcm}^{-1}) = 59.05 + 1.84B + 2.03F + 3.16I. \]

This equation showed the magnitude of different independent variables. The positive effect of various components (yeast extract, inducer and FeSO₄·7H₂O) was further investigated by applying a central composite design. **Optimum level determination by a central composite design (CCD).** For the determination of the optimum level and combined effect of yeast, inducer and FeSO₄·7H₂O, the central composite design, given in Table 2, was used, and a second-order polynomial equation was derived to explain the dependence of an amide hydrolyzing enzyme for biotransformation of *N*-substituted aromatic amides.
on the different medium components. The results of the CCD were fitted into a second-order polynomial equation for the prediction of response on the bases of coded value:

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ANOVA analysis of the CCD results was performed and a four-process order was suggested by Design expert 8.0. A quadratic process order proved to be the best due to a high standard deviation (5.54), a high R-squared value (0.90), and a high press value (19134.91), and was processed for further analysis (Table 3). The "Pred R-Squared" of 0.78 is in reasonable agreement with "Adj R-Squared" of 0.80. The analysis of the variance of the quadratic regression model suggested that the model was very significant as was evident from the correlation coefficient (0.94)—closer to 1 denotes a better correlation between the observed and predicted responses. The coefficient of variation (CV) indicates the degree of precision with which the experiments were compared. A lower reliability of the experiment is usually indicated by a high value of CV. In the present case, a low CV (7.25) denotes that the experiments performed were highly reliable (Table 4). 3D graphs were generated for the regression analysis of a CCD design of a pairwise combination of three factors for the production of ATA. These 3D response surface plots (Figs. 5a, b, and c) described the effects of the independent variables and the combined effects of each independent variable upon the response.
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Validation of model. The maximum activity experimentally obtained, 94.45 ± 0.02 U/mgdcm⁻¹, was closely related to the predicted value 93.82 ± 0.03 U/mgdcm⁻¹ calculated by the ANOVA analysis. The perturbation plot (Fig. 6) shows the optimum value for the variables: yeast extract: 0.125 g; inducer: 0.3% (30 mM); and FeSO₄·7H₂O: 0.033 g, per litre. The model was validated by performing the experiments under optimum conditions, which resulted in 95.52 ± 0.03 U/mgdcm⁻¹; hence, proving the validity of the model.

Multiple feeding of the inducer. Different concentrations of isobutyronitrile as inducer were fed at different times and the induction of ATA of amidase was assayed. Feeding of 20 mM isobutyronitrile (100 mM stock) after regular time intervals in Set 2 produced the maximum ATA (100.64 ± 0.03 U/mgdcm⁻¹) with a reasonable cell biomass, i.e. 6.5 mgml⁻¹, and the feeding of a higher percentage of inducer retarded the growth of the organism. In high exponential level feeding of isobutyronitrile in 5 different sets, the induction of ATA of amidase was observed, but not to the extent as in the case of a constant level of inducer. This might be due to the toxicity of the high level of inducer. The feeding of a higher concentration of isobutyronitrile in the growth medium (Set 5) stopped the growth of the organism. In the case of a low exponential level of feeding, the maximum induction was observed in Set 5 (80.34 ± 0.04 U/mgdcm⁻¹), while, in the case of a decreased feeding level, the maximum ATA was obtained in Set 1. The low feeding of the inducer resulted in less growth as well as a lesser ATA of amidase. A similar effect was observed when feeding a lower concentration of inducer for Set 1 with a constant, high and low exponential level, and Set 5 with a decreasing level. After 56 h of incubation, the production of the ATA of amidase was decreased in all levels which might be due to the accumulation of secondary metabolites and the production of toxic substances.

ATA of amidase from Pseudomonas putida BR-1 for the catalysis of different amides. The ATA of this organism was used for the biotransformation of a variety of aliphatic, aromatic, enantioselective amides with hydroxylamine to corresponding hydroxamic acids. Short chain amides (acetamide and propionamide) were transformed rapidly. Among the unsaturated amides, acrylamides and methacrylamide resulted in a good ATA of amidase from Pseudomonas putida BR-1. Amides having a bulky side chain (L-tryptophanamide and D,L-phenylglycinamide) were found to be poor substrates for ATA. Acetamide has the highest ATA (123.30 ± 0.02 U/mgdcm⁻¹) among the aliphatic amides. In the case of the aromatic amides, benzamide gave (66.24.30 ± 0.04 U/mgdcm⁻¹) ATA; while, among the N-substituted aromatic amides, nicotinamide resulted in the maximum ATA (100.64 ± 0.03 U/mgdcm⁻¹).

Discussion

The present study was focused on investigating the microflora from the rhizosphere of cyanogenic plants for the conversion of amides and hydroxylamine to hydroxamic acids. Enrichment isolation techniques resulted into a highly active bacterial isolate BR-1, which
was finally identified as \textit{Pseudomonas putida} BR-1. To utilize the maximal potential of a microbe for the production of a desired enzyme, physiological conditions play a very crucial role; thus, it is very important to optimize the culture parameters for enhancing the microbial production of the desired product (Krieg et al., 2002). A number of different amide-degrading microorganisms have been earlier isolated from soil, but the isolate BR-1 exhibited a very high ATA for \textit{N}-substituted aromatic amides, i.e. 100.64 ± 0.03 \text{Umgdcm}^{-1}, as compared with 9.9 \text{Umg}^{-1}, 24 \text{Umg}^{-1} and 16.1 \text{Umgdcm}^{-1} from the purified amidase of \textit{R. rhodochrous} R312, \textit{Paracoccus} sp. M1 and whole cells of \textit{Bacillus smithii} strain ITR6b2 (Agarwal et al., 2013; Fournand et al., 1998; Shen et al., 2012). \textit{Pseudomonas putida} BR-1 efficiently grew in a mineral salt medium (M5) resulting in considerable biomass yield, as well as the production of ATA. Tryptone acted as a very good source of carbon and nitrogen for the production of ATA in \textit{Pseudomonas putida} BR-1, while for \textit{Geotrichum} sp. JR1, acetonitrile has been reported to be a suitable carbon source (Rezende et al., 2004). In general, the ATA of amidase is produced constitutively by many microbes, whereas in some microorganisms it is induced by amides and nitriles (Pal and Samanta, 1999). The ATA of \textit{Pseudomonas putida} BR-1 is also inducible in nature, and isobutyronitrile (0.3\% \text{v/v}) proved to be the best inducer for the production of ATA. The growth of \textit{Pseudomonas putida} BR-1 partially decreased at a higher concentration of inducer, which might be due to the toxicity resulting in a decline of ATA production. A higher level of inocula of \textit{Pseudomonas putida} BR-1 showed a low ATA that might be due to an early depletion of the inducer. In \textit{P. aeruginosa} 8602A strain, a maximum amidase production was achieved using 0.5\% (\text{v/v}) of inoculum (Brammar and Clarke, 1964). The amidase of \textit{Pseudomonas putida} BR-1 exhibited a maximum ATA when the production medium was supplemented with 5\% inoculum. Sharma et al. (2011) reported a 20 h incubation in the case of a \textit{Geobacillus pallidus} BTP5X MTCC 9225 culture for amidase production, while this organism produced a maximum ATA after 56 h of incubation (58.77 ± 0.03 \text{Umgdcm}^{-1}), and the further increase in OD at 600 nm might be due to the production of polysaccharides in the late stationary phase (Celik et al., 2008). Most of amidase-producing organisms which exhibit ATA grow and produce enzyme around a neutral pH (Krieg et al., 2002); however, this organism efficiently produced biomass and ATA at a slightly alkaline pH (8.5). \textit{Pseudomonas putida} BR-1 produced a maximum ATA at 25°C, whereas an optimum production of amidase has been observed at 30°C and 50°C by Fournand et al. (1998) and Makhongela et al. (2007), respectively. This organism was thus mesophilic in its requirement of temperature for the growth and production of ATA. In order to evaluate the cumulative effect of different operational variables and their respective influence on the response, it is very important to use an experimental design that could account for these interactions (Bhatia et al., 2013b; De Coninck et al., 2004; Xiao et al., 2007). Therefore, the optimization of culture conditions was carried out using response surface methodology (RSM), which resulted in a 25\% increase in the production of the ATA of amidase. Multiple feeding of the inducer is a better approach to harness the maximum output of the microorganism for enzyme production rather than a single-time feeding of a higher amount of inducer, which might be toxic. With multiple feeding of the inducer at a constant level, a maximum ATA (100.64 ± 0.03 \text{Umgdcm}^{-1}) was achieved, while Sharma et al. (2011) reported a maximum production of ATA when the inducer was fed only once for the induction of a similar type of enzymatic system. Like most of the mesophilic amidases which rapidly lose their activity above 50°C, \textit{Pseudomonas putida} BR-1 also lost its ATA in 1 h at 60°C, but retained its ATA for a long time when stored at 4°C. The ATA of \textit{Pseudomonas putida} BR-1 was higher for \textit{N}-substituted aromatic amides among the hitherto reported amidase systems, and this has a potential application in the biotransformation of a number of aliphatic, aromatic and \textit{N}-substituted aromatic amides with hydroxylamine to corresponding hydroxamic acids.

The present study has reported a higher ATA organism, \textit{Pseudomonas putida} BR-1, which is capable of biotransforming a variety of amides into useful hydroxamic acids. RSM has been shown to be an important statistical tool for the optimization of process parameters for the ATA of amidase by \textit{Pseudomonas putida} BR-1. This amidase showed the highest ATA (100.64 ± 0.03 \text{Umgdcm}^{-1}) of the \textit{N}-substituted aromatic amides among the hitherto reported enzymes. The optimization of culture conditions led to a 16-fold increase in the production of ATA by \textit{Pseudomonas putida} BR-1. This enzyme showed a better thermostability at a high temperature in comparison with enzymes reported from mesophilic systems, and it shows a very good potential for the synthesis of industrially important \textit{N}-substituted aromatic hydroxamic acids, as well as for the bioremediation of an amide/nitrile-contaminated site.

Acknowledgments

The authors are highly grateful to the University Grant Commission (UGC) New Delhi, India, and the Department of Biotechnology (DBT), India, for providing financial assistance in the form of SRF to Ravi Kant Bhatia, Shashi Kant Bhatia and Praveen Kumar Mehta, respectively. Computer facilities provided by the Bioinformatics Center of Himachal Pradesh University are duly acknowledged.

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