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Full Length Research Paper

Amidase from plant growth promoting rhizobacterium

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Ninety three cultures of plant growth promoting rhizobacteria (PGPR) isolated from rhizosphere of *Pisum sativum*, among them one isolate identified as *Pseudomonas putida* was found to be potential amidase producer. The organism exhibited a battery of PGPR traits including enhanced production of plant growth hormone indoleacetic acid (IAA) and siderophore. *P. putida* MTCC 6809 exhibited both intracellular and extra-cellular amidase activity. The organism produced maximum extracellular amidase enzyme at 30°C and pH 7.5 in shaking state. The organism hydrolyzed a wide range of aliphatic amides that included acetamide, propionamide, acrylamide and butyramide. Acrylamide is a known carcinogen, teratogen and neurotoxicant and utilization of acrylamide by *P. putida* MTCC 6809 assume great importance. The organism is also tolerant to number of heavy metals at higher levels. These characteristics make *P. putida* MTCC 6809 an excellent candidate for field application in contaminated soil.

Key words: PGPR, amidase, aliphatic amides, acrylamide, heavy metal tolerance.

INTRODUCTION

Amidases (acylamide amidohyrolases EC 3.5.1.4) catalyze the hydrolysis of the carboxylic amide bonds to liberate carboxylic acid and ammonia (Asano and Lubbehusen, 2000). They are widely distributed in nature and are involved in nitrogen metabolism in both prokaryotic and eukaryotic cells. The ability of amidase to convert the cyanofunctionally into either an amide or an acid, together with regio and stereoselectivity, makes it a good biocatalyst in biotransformation processes. Amidases have been as biocatalysts for the production of ammonium, acrylate and acrylic acid, compound of intense industrial use (Nagasawa and Yamada, 1989).Apart from amide hydrolysis activity, some amidases also exhibit acyl transferase activity (Maestracci et al., 1980). Furthermore, many of these amidases have dual specificities, cleaving amide as well as ester and nitrile bonds (Patricelli and Cravatt, 2000; Pollmann et al., 2003; Cilia et al., 2005). Amidases form an integral part of nitrile converting enzymes and is known to have versatile industrial applications. In addition to several industrial applications, amidase plays an important role in degradation of agricultural and industrial wastes (Shanker et al., 1990).

Enzyme activity is considerably greater in the rhizosphere of plants than in bulk soils which often lends specific advantage to the plant and this activity is due to either a specific flora or the plant root or due to both (Curl and Truelove, 1986; Zahir et al., 2001). Many of the enzymes detected in soil are hydrolases and amidase is one of the important among them. Apart from amide hydrolysis activity, amidases have been shown to be involved in the biosynthesis of indole acetic acid (IAA) an important plant hormone (Pollmann et al., 2003; 2006; Spaepen et al., 2007). During the interaction between IAA-producing bacteria and plants, this phytohormone is used by bacteria as part of their colonization strategy, including phyto-stimulation and circumvention of basal plant defense mechanisms (Spaepen et al., 2007). Hydrolysis of carboryl based pesticides by soil amidases is reported by Kay-Shoemake and co-workers (2000).

Many investigations reported elevated levels of amidase enzyme in soil samples. Owing to the versatile applications, we screened PGPRs isolated from the rhizosphere of *Pisum sativum* that could be a potent source of amidase enzyme. Along with screening, parametric optimization for maximum amidase production is presented here in a promising PGPR strain of *Pseudomonas putida* MTCC 6809. The bacterium could also be the source to

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develop desired microbial bioaugumentation for growth of field crops in contaminated soil.

MATERIALS AND METHODS

Microorganisms

Ninety three isolates of PGPR obtained from rhizosphere of *P. sativum* comprising strains of *Pseudomonas* spp. (23), *Bacillus* spp. (19), *Rhizobium* spp. (34) and *Azotobacter* spp. (17) were included in the study.

An amidase producing strain *E. coli* NCIM 2568 was obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India which was included in the study as control.

Screening of amidase producers

A qualitative estimation of amidase producing organism was done using a modified method by Rahim et al., 2003. Screening medium contained the following composition (g/l). K_2HPO_4 , 3.5; KH_2PO_4 ,2.2; $MgSO_4$, 0.05; 0.5ml of trace element solution containing (mg/l) $ZnSO_4.7H_2O$,0.3; $FeSO_4.7H_2O$,0.6; $MnSO_4$,0.3; $CuSO_4.5H_2O$, 0.4; $CaCl_2.2H_2O$, 0.5g/l Phenol red 0.012; Agar,15 and acetamide 20 (as sole carbon and nitrogen source). Acetamide was filter sterilized using Axiva membrane filter assembly and was added to the autoclaved medium. The pH of the medium was adjusted to 6.6 ± 0.2. Different PGP organisms were inoculated into the broth/ agar plates and then incubated for 36 h at 30°C. The broth and plates were regularly observed for colour change that is, from yellow to pink. A potent amidase producing organism was selected for further studies.

Identification of the organism

The promising amidase producer was identified to species level by Microbial Type Culture collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and assigned accession number MTCC 6809.

Determination of PGP characters

Ammonia production: The organism was grown in peptone water at 30°C for 4 days and 1 ml of Nessler's reagent was added. Production of ammonia was represented by development of faint yellow colour (Bakker and Schippers, 1987).

Indole acetic acid

The organism was inoculated in LB medium amended with 50 mg ml^{-1} of tryptophan and incubated at 30°C for 24 h. After incubation, 2ml of cell suspension was centrifuged at 1000 rpm for 10 min and 2 - 3 drops of orthophosphoric acid was added to the supernatant along with 4 ml of Solawaski's reagent (50 ml 35% perchloric acid; 1 ml of 0.5 M $FeCl_3$). The tubes were kept at room temperature for 20 min. IAA production was indicated by the development of pink colour. Quantification of IAA was estimated at OD 530 nm using standard IAA graph (Bric et al., 1991).

Catalase production

Bacterial culture was grown in a nutrient agar medium for 18 - 24 h. The cultures were mixed with appropriate amount of H_2O_2 on a glass slide to observe the evolution of oxygen.

Phosphate solubilization

The isolate was tested for phosphate solubilizing ability on Pikovskaya medium (Pikovskaya, 1948) incorporated with tricalcium phosphate (Ca_3 $(PO_4)_5$). Phosphate solubilization was indicated by the formation of a clear halo zone around the bacterial growth after 3 days of incubation.

HCN production

The isolate was streaked on King's B medium amended with 4.4 gl^{-1} of glycine. The plates were covered with sterile filter paper impregnated with 0.5 % picric acid in 2 % sodium carbonate, sealed with parafilm and incubated for 4 days (Bakker and Shippers, 1987). Development of yellow colour on the filter paper indicates the positive result.

Siderophore production

Siderophore production was detected by the universal method of Schwyn and Neilands (1987) using blue agar plates containing the dye Chrom Azurol S (CAS). Orange halos around the colonies on blue were indicative of siderophore production.

Analytical methods

The soluble proteins were estimated by using the method given by Lowry et al., 1951. Cell growth was monitored by determining the optical density of the culture medium at 540 nm using (Genesys6, Thermoelectron) spectrophotometer, the culture was diluted if necessary. Amidase activity was determined by measuring the ammonia released (Nesslerization reaction) spectrophotometrically at 450 nm (Imada et al., 1973). One unit of amidase activity is expressed as the amount of enzyme required to release 1 µmols of ammonia per minute under the assay conditions.

Intracellular amidase production

To check the intracellular amidase production, cells were harvested at different time intervals (8 - 36 h) by centrifuging at 10,000 rpm for 15 min in Sigma centrifuge which was then stored at 4°C for 30 min. 5 gm cells were then dissolved in 20 mM PO_4 buffer pH 7.4 containing 10 mM EDTA. Lysozyme was dissolved at a concentration of 0.5 mg/ml. The mixture is then stirred gently for 30 min at 4°C and 0.1 mg of DNase and 50 µl of 0.2 M $MgCl_2$ were added to it. The mixture was incubated for 15 min and centrifuged at 20,000 rpm for 30 min at 4°C. The clear supernatant was used for enzyme assay.

Parametric optimization for enzyme production

Different physiological (pH of the production medium, temperature of incubation and incubation time) and nutritional parameters (substrate, carbon and nitrogen source, concentration etc.) were optimized for maximum amidase production.

Heavy metal tolerance test

Tolerance to metal ions was determined by minimal inhibitory concentration (MIC) technique by agar dilution method (Luli et al., 1983). Agar plates supplemented with different concentrations of heavy metals (50 - 250 µg/ml) were inoculated aseptically by exponentially growing culture of bacteria. The plates were incubated for 36 h at 30°C, growth of the isolates indicated positive tolerance. The metal used were As, Co, Cd and Cr, Zn, Ni and Cu and Hg. The compounds used were As_2O_3, $CoCl_2$, Cd $(NO_3)_2$, $K_2Cr_20_7$, $ZnCl_2$, Ni $(NO_3)_2$, $CuCl_3$, and $HgCl_2$.

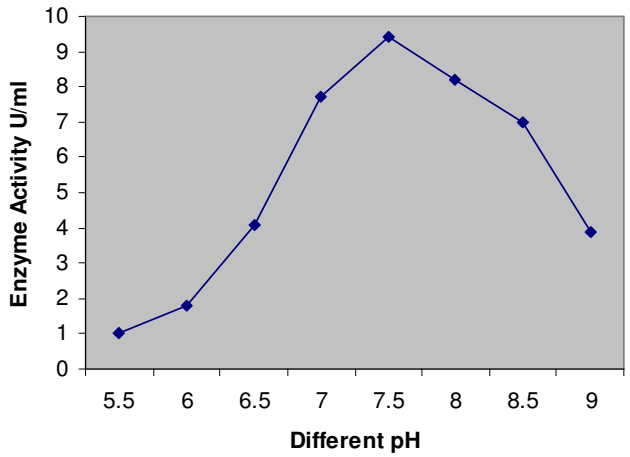

Figure 1. Effect of pH on amidase production.

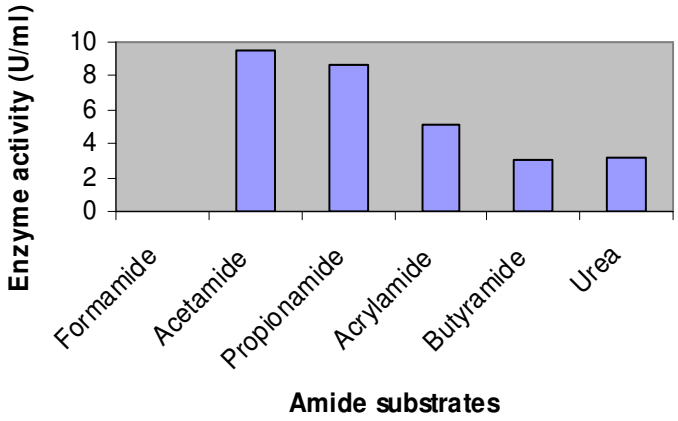

Figure 2. Utilization of substrates in enzyme production.

Plasmid curing

The microorganism was subjected to acridine orange mediated plasmid elimination (Ramteke, 1997). Overnight logarithmically growing culture was inoculated in nutrient broth containing acridine orange 20 µg ml^{-1} incubated at 30°C for 48 h. After incubation the culture was serially diluted and plated in nutrient agar plates. Individual colonies were screened for plasmid-encoded traits.

RESULTS AND DISCUSSION

Among the various PGPR isolated from rhizosphere of *P. sativum*, one isolate identified as *Pseudomonas putida* was found to be potential amidase producer. The pH of the medium was found to increase from 7.0 - 8.3 after 48 h of incubation as ammonia was released due to amide hydrolysis. Since intact cells released ammonia into the medium so the enzymes could be assayed *in situ* (Miller and Gray, 1982). The organism is deposited in Microbial Type Culture Collection (MTCC), India and assigned accession number MTCC 6809.

The organism exhibited a battery of PGPR characteristics. It showed reasonably good production (4.2 µg/ml) of plant growth hormone indoleacetic acid (IAA) and siderophore and was also found positive for the production of ammonia and catalase. The bacterium exhibited stimulation of root and shoot growth in presence of heavy metals (data not shown). Amidases are known in biosynthesis of IAA (Pollmann et al., 2006; Spaepen et al., 2007). Thus, amidase producing bacteria in the plant root rhizosphere that can rapidly convert precursors to biologically active molecules holds promise for increasing crop production.

The enzyme production is greatly affected by pH; hence production medium with different pH was used for amidase production and was assayed for enzyme activity (Figure 1). Amidase production was optimum at pH 7.5. Also the amidase activity due to various pH was found to be statistically significant (SE (m) 0.026; C. D. 0.08).

P. putida MTCC 6809 exhibited both intracellular and extracellular amidase production. However, production of intracellular enzyme was three fold less as compare to extracellular enzyme. The organism produced maximum extracellular enzyme activity after 12 h of incubation at 30°C and pH 7.5 in shaking state.

Utilization of various amide substrates were screened for amidase production (Figure 2). The organism hydrolyzed a wide range of aliphatic amides that included acetamide, propionamide, acrylamide and butyramide. But the organism was not able to hydrolyse, carbon compound formamide. Among the amides tested, acetamide followed by propionamide was found to be the better substrates.

Acetamide and propionamide were further studied to obtain its optimum concentration in enzyme production. Acetamidase activity at 2% w/v concentration was found to be statistically significant (SE (m) 0.026; C. D. 0.082) (Figure 3).

On the other hand the organism produced maximum enzyme at 1 % w/v concentration of propionamide (Figure 4). Also at higher substrate concentrations [3% and 4% (w/v)] it was observed that there was no increase in enzyme production. This might be due to less conversion of amide substrates into products or hydrolysis of the amide compounds to products due to increased amide concentration. Brammer and Clark (1964) reported that amidase production by *P. aeruginosa* was repressed at higher concentration of acetaamide which was used as inducer, as at higher concentration the system was saturated by the inducer.

Amides when used as sole source of carbon and nitrogen, growth of the organism was observed to be less and therefore, the production medium was supplemented with various sources of carbon and nitrogen to enhance both growth and enzyme production. Among the various carbon sources tested glucose followed by fructose emerged the best carbon sources (Figure 5). Lactose was the least suitable carbon source. Enhanced production of enzyme was observed when glucose concentration was at 1.0% (w/v) but there was a significant decrease in enzyme pro-

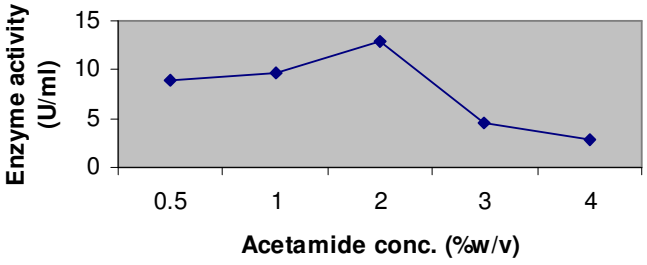

Figure 3. Effect of concentration of acetamide on amide production by *Pseudomonas putida* MTCC6809.

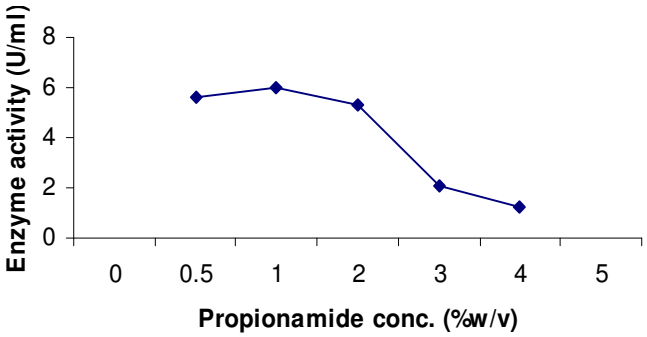

Figure 4. Effect of concentration of propionamide on amidase production *Pseudomonas putida* MTCC6809.

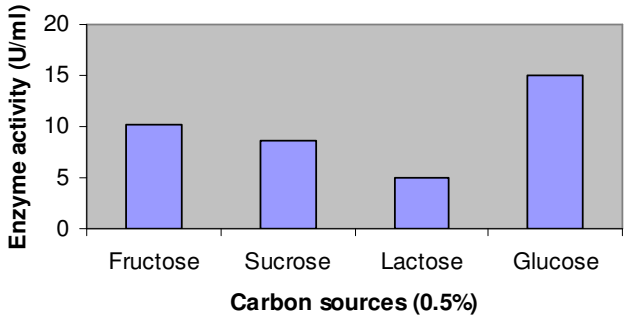

Figure 5. Effect of carbon sources on amidase production.

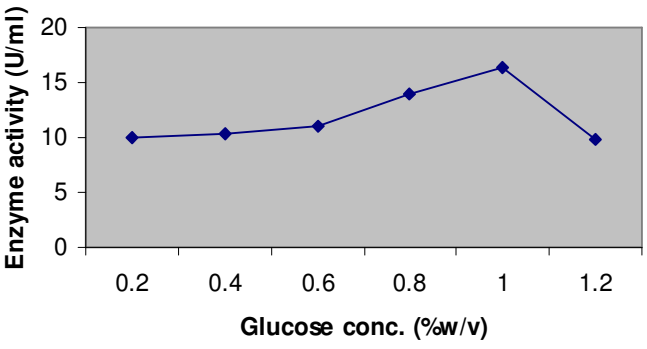

Figure 6. Effect of glucose concentration on amidase production.

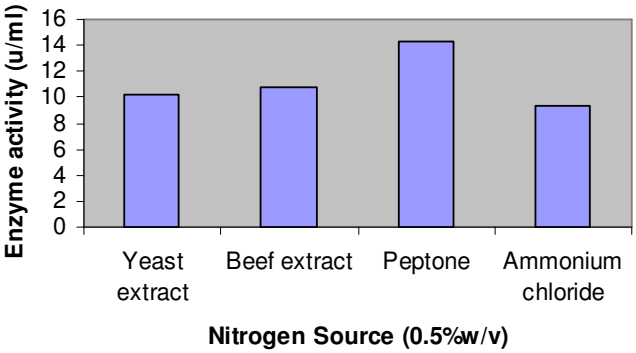

Figure 7. Effect of nitrogen sources on amidase production.

duction when the glucose concentration was raised to 1.2% (w/v) suggesting that the enzyme production was repressed by higher concentration of glucose (Figure 6).

Catabolic repression of amidases by common sugars was also observed in other organisms. Fructose in *Alcaligenes eutrophus* (Friedrich and Mitrenga, 1981) and glucose in *Pseudomonas* spp (Shanker et al., 1990) were found responsible for catabolic repression of amidases. However, contradictory to these reports we observed that production of amidase in *P. putida* MTCC 6809 is not repressed catabolically by fructose and glucose. Also, we checked the various nitrogen sources to enhance amidase production, among them peptone enhanced production of the enzyme (Figure 7).

Bacterial resistance to metal ions seems to be directly related to the presence and adopted response to these elements in the environment (Timoney et al., 1978). Thus, tolerance to heavy metals gives an added advantage of survival in the polluted environment. *P. putida* MTCC 6809 was isolated from the sewage irrigated rhizosphere and was found tolerant to higher concentration multiple heavy metals (Table 1).The organism showed tolerance to high levels (>100 $ugml^{-1}$) of Ni, Pb, Zn and As. Tolerance to Co, Cr, Cu and Cd was at 50 $ugml^{-1}$. However, surprisingly we observed that tolerance to heavy metals were found not curable when acridine orange was used as curing agent suggesting chromosomal traits. Acrylamide is used in numerous industrial processes but its extensive and discriminate usage has lead to the contamination of terrestrial ecosystem. Acrylamide is a known carcinogen, teratogen and neurotoxicant (Dearfield et al., 1988; Klause and Schmahl, 1989). In this context, utilization of acrylamide by *P. putida* MTCC 6809 assumes greater importance. This finding is very important from the point of environmental detoxification of acrylamide.

Polyacrylamide (PAM) is currently used as an irrigation water additive to reduce the amount of soil erosion that occurs during furrow irrigation of crops. Elevated soil amidase activity specific toward the large PAM polymer has been reported in PAM-treated field soils (Kay-Shoemake et al., 2000). Additionally, nitrile compounds are dis-

Table 1. Tolerance to heavy metal.

Heavy metal	Conc. µg/ml	Tolerance
Nickel	200	+
Lead	200	+
Arsenic	200	+
Zinc	100	+
Cobalt	50	+
Copper	50	+
Chromium	50	+
Cadmium	50	+
Mercury	50	+

charged into the environment as industrial waste water, agricultural chemicals, etc. In mineralization of nitriles and corresponding amides, amidases play an important role in addition to nitrile-hydrolyzing enzymes (Asano et al., 1982; Shanker et al; 1990). Hydrolysis of carbaryl by a soil amidase was also demonstrated by Kay- Shoemake and coworkers (2000).

Thus, considering overall characteristics to produce amidase, plant growth promoting traits and to tolerate higher levels of heavy metals makes *Psuedomonas putida* MTCC 6809 an excellent candidate for field application in contaminated soil.

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