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Shake flask studies for the production of penicillin G acylase from *Aspergillus niger*

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ABSTRACT

Thirty different strains of fungi were isolated from local habitats. The cultures were grown aerobically under submerged condition in 250 ml shake flasks containing 50 ml basal media. Lactose was used as a source of carbon. The enzyme activity was determined by quantification of 6-amino penicillanic acid in the reaction mixture. After screening and identification of 12 fungal strains, only one strain of *Aspergillus niger* (17-M) was showing the highest activity of enzyme (40 IU/ml). Out of seven different carbon sources studied, 0.4% lactose was found as the best carbon source, which exhibited the highest enzyme activity (48 IU/ml) at pH 5.5. The conversion rate of 3% and 5% potassium salt of benzyl penicillin by penicillin G acylase of *A. niger* (17-M) was 35% and 26%, respectively. Km value (0.82 mM) was promising in terms of better enzyme production.

Key words: ***Aspergillus niger*, penicillin G acylase, phenyl acetic acid, 6-amino penicillanic acid.**

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INTRODUCTION

Penicillin G acylase (PGA) finds commercial application for the hydrolysis of penicillins to give carboxylic acids and 6-amino penicillanic acid (6-APA). The enzyme has been widely demonstrated in bacteria, yeasts, and filamentous fungi[2,10,15,16]. The PGA has been given different names by various workers[1,17]. It hydrolyzes phenoxy methyl penicillin much more readily than benzyl penicillin[24]. Interest in 6-APA came after the successful efforts of organic chemists in acylating the molecule to produce various semi-synthetic penicillins, such as ampicillin and amoxicillin[8]. However, economic, environmental and operational advantages of the enzymatic process over the chemical route have now been found with the preparations of purified enzyme, and the bulk of 6-APA produced today is by enzymatic deacylation of penicillin G or penicillin V[6].

Considerable work has been done on bacterial acylases[18]. However, research report on fungal mycelium acylases is very limited. Pakistan being an agricultural country has potential resources of biomass, such as molasses, corn steep liquor, cheese whey, distillery wastes and defatted oil seed cakes, for their exploitation in the manufacture of cell biomass, enzymes or microbial metabolites[19]. So, the present research work is based on shake flask studies to screen mould strains for the production of penicillin G acylase (PGA).

MATERIALS AND METHODS

Isolation and identification. Soil samples were collected from around PCSIR (Pakistan Council of Scientific and Industrial Research Laboratories), Lahore, Karachi, Peshawar and Quetta campuses. The PGA producing fungi were isolated from different soil samples according to the method described earlier[13], and were characterized by Pakistan Type Culture Collection (PTCC). The cultures were examined under low power microscope after mounting with lactophenol cotton blue dye. The morphological and physiological characteristics showed that only twelve strains belong to *Aspergillus niger* Tiegh. The strains were transferred on nutrient agar slants, incubated by seven days to 30 C, and stored to 4 C. Each value of the experimental data is an average of three replicates.

Fermentation technique. Shake flasks (250 ml) containing 100 ml of basal medium, consisted of (composition per liter): glucose, 2 g; poly-peptone, 5 g; yeast extract, 5 g; K_2HPO_4, 1 g; and $MgSO_4 \cdot 7H_2O$, 0.2 g. Flasks were used in triplicate. In the successive experiment, the 4% w/v lactose was used as a carbon source instead of glucose. Initially, the pH of the medium was adjusted to 5.5 with hydrochloric acid (0.1 M per liter). After sterilization at 121 C for 15 min, the flasks were cooled and inoculated at 3% v/v with 0.5 ml of a suspension of *A. niger* (17-M), prepared by adding 5 ml sterile saline to a working agar slope. The flasks were shaken at 250 rpm for 24 h at 30 C in a rotary shaker. After 20 h incubation, 10,000 units of phenyl acetic acid (PAA) were added as an inducer of PGA, and cultivation was continued for further 4 h. Mycelial colonies were removed by filtration. The liquid fraction was dialyzed and PGA activity was determined.

Enzyme reaction. The activity of penicillin G acylase was determined by measuring the amount of 6-APA

produced in a reaction mixture containing approximately 100 mg mycelium (10 mg/ml), and 100 mg benzyl penicillin per ml in 0.1 M tris buffer at pH 7.0. The reaction mixture was mixed thoroughly, and shaken at 30 C for one hour. At the end of the incubation period, the suspension was instantly treated with 0.5 ml of formic acid (98%) and centrifuged at 10,000 rpm for 20 min. The supernatant of each reaction mixture was collected separately. To 2 ml of each supernatant was added 1 ml citrate buffer and 0.5 ml ninhydrin solution, and PGA activity was determined[11,12]. After 25 min, absorption at 410 nm was read. Standard solution of 6-APA was assayed whenever the hydrolytic activity was determined. One unit of the enzyme activity was defined as the amount of enzyme required to produce one micromole of 6-APA per hour.

Determination of fungal biomass. Mycelial colonies were obtained from culture broth by centrifugation, and were dried at 105 C to constant weight.

Isolation and purification of 6-APA. The enzyme penicillin G acylase (PGA) derived from the mycelial suspension of *A. niger* (17-M) was separated and purified according to the modified method of Okachi *et al*.[14], in which $Ba(OH)_2$ was replaced with $Ca(OH)_2$.

Kinetic parameters. Km and Vmax values were determined by computer regression analysis. The data was obtained by using the substrate concentration (benzyl penicillin) with the velocity in micromole $minute^{-1}$. Four experiments were performed for each substrate concentration. The dependence of initial rate on substrate concentration was measured, and the experimental data was determined by double reciprocal plot.

Statistical analysis. The experiments were carried out in triplicate, and standard deviations were calculated according to the method of Steel *et al*.[23]. Multivariate Anova was used to determine the significant differences ($p<0.05$).

RESULTS AND DISCUSSION

Screening and selection. Thirty cultures of fungi were isolated from soil sample taken from various agricultural soils of PCSIR. Only 12 strains of mould were found potent for the production of extra-cellular enzyme PGA (**Table 1**). The highest enzyme productivity of isolate No. 17-M was 40 IU/ml, which was selected for further studies.

Strain identification. The strain 17-M showed green colonies, finely roughened stalks, and spore bearing heads, which

Table 1. Extra-cellular enzyme (PGA) producing strains of *Aspergillus niger* in 250 ml shake flasks.

Isolate No.	Activity (IU/ml)	Fungal biomass (g/l)	Specific activity (IU/mg of total protein)
5-M	5±0.55	2.19±0.12	17±1.12
7-M	9±0.63	3.2±0.09	22±0.59
9-M	12±0.52	3.5±0.09	39±0.78
11-M	15±0.93	3.9±0.17	43±0.57
13-M	21±0.62	4.0±0.10	57±0.73
14-M	37±0.33	4.5±0.13	75±0.80
17-M	40±0.71	5.2±0.15	83±0.70
21-M	33±0.42	4.6±0.07	68±0.83
25-M	25±0.39	4.9±0.13	59±0.80
27-M	19±0.44	3.5±0.07	45±0.83
29-M	15±0.32	2.7±0.11	38±0.13
30-M	11±0.21	1.5±0.10	25±0.08

were large tightly packed, globular and black. The conidia were rough, with bands of pigment. Most of them had sclerotia of buff to gray color. Taxonomic comparisons were made with the help of Dermatophyte Identification Scheme[7]. Optimum temperature for growth was 30 C and the pH was 5.5. The microbiological properties of the isolate were similar to those of *A. niger*, which were confirmed by Frazier and Westhof [4] and Collins and Layne[3]. In fact, it has been reported that some strains of *Aspergillus* spp. produced PGA[5].

Effect of carbon sources on PGA production. The composition of culture media and cultivation condition exert great effect on the synthesis of enzymes. For example, carbon nitrogen ratio and other growth factors greatly influence the fermentation pattern[8]. Seven different carbon sources, such as lactose, starch, glucose, sucrose, fructose, raffinose, and cellubiose, at the level of 0.2%, 0.4% and 0.6% were evaluated into the fermentation medium for the maximum production of the enzyme by strain 17-M of *A. niger*. The results are given in **Table 2**. All the sugars tested supported fungal growth. However,

Table 2. Effect of carbon sources on fungal growth and PGA production by *Aspergillus niger* (17-M).

Carbon sources	Concentration (%,w/v)	Activity (IU/ml)	Mycelial mass (g/l)	Specific activity (IU/mg of total protein)
Lactose	0.2	37±0.55	3.19±0.12	77±1.12
	0.4	48±0.63	4.5±0.09	92±0.59
	0.6	40±0.52	3.5±0.09	89±0.78
Starch	0.2	41±0.93	3.9±0.17	73±0.57
	0.4	43±0.62	3.0±0.10	84±0.73
	0.6	46±0.71	3.2±0.15	81±0.70
Glucose	0.2	39±0.33	3.5±0.13	85±0.80
	0.4	32±0.42	3.6±0.07	78±0.83
	0.6	35±0.39	3.9±0.13	79±0.80
Sucrose	0.2	21±0.44	2.5±0.07	75±0.83
	0.4	29±0.32	2.7±0.11	68±0.13
	0.6	25±0.65	2.23±0.13	65±1.25
Fructose	0.2	39±0.70	3.03±0.21	59±1.39
	0.4	33±0.73	3.41±0.23	62±1.21
	0.6	38±0.65	3.45±0.15	66±1.35
Raffinose	0.2	21±0.87	3.56±0.29	60±1.43
	0.4	25±0.69	2.89±0.25	63±1.55
	0.6	29±0.49	2.93±0.13	71±1.23
Cellubiose	0.2	41±0.49	3.23±0.13	75±1.23
	0.4	35±0.69	3.45±0.24	77±1.19
	0.6	37±0.43	3.25±0.18	78±1.09

the most suitable sugar for enzyme production was lactose at the concentration of 0.4% (w/v), where the highest enzyme activity was recorded (48 IU/ml). Glucose at the concentration of 0.4% also enhanced considerably the enzyme production. In the subsequent experiment, 0.4% lactose was used as a carbon source, and the pH of the medium was adjusted to 5.5. The results, which are in accordance with the data obtained previously with PGA from other sources, showed that some strains of *Aspergillus* produced acceptable enzyme levels when grown on lactose as source of carbon. Yang *et al*.[26] reported that a recombinant *B. subtilis* having the pac gene of *B. megaterium* produced more enzyme (PGA) by the addition of sucrose or starch in the medium. Senthivel and Pai[21] reported that the production of PGA was increased by sucrose and repressed by glucose and fructose. In *E. coli*, the synthesis of PGA was repressed by glucose, fructose, maltose and glycerol[22]. In *Penicillium chrysogenum*, lactose and mannitol gave the slowest rate of growth of mycelia[20]. In our studies, lactose enhanced the enzyme production. Addition of glucose or fructose did not repress the PGA production by *A. niger*. The relationship between lactose and PGA production shows that PGA was produced at low concentration (0.4%). In order to produce the enzyme earlier, the lactose in poly-peptone medium was reduced to 4 g/l, which is the level that *A. niger* actually needs for growth. After that, we may expect that PGA will be produced earlier and the fermentation process terminated within 30 h, instead of 50-70 h.

Effect of time course on enzyme production in shake flask. The rate of fermentation was studied in 250 ml shake flasks. The enzyme production initiated slowly after the inoculation. It increased gradually up to 24th h (**Fig. 1**). A drastic increase was found between 20th and 24th h of incubation, which shows that the production of enzyme is related to fungal growth. Similarly, our results are in accordance with the findings of Shewale and Sivaraman[22]. Liu *et al*.[9] also reported that maximum enzyme activity occurred after 20 h and 24 h of incubation with *A. niger*.

Hydrolysis of phenyl acetic acid (PAA) by A. niger *(17-M)*. The mycelial suspension of *A. niger* (17-M) was cultured in nutrient medium containing 0.15% PAA at 30 C. After 72 h, the mycelial colonies were collected, washed, and suspended in distilled water. Potassium salt of benzyl penicillin was added to the mycelial suspension at the concentration of 3% and 5%, under continuous stirring, and pH control of 7.0 with 2 N NaOH. The conversion rate of 3% and 5% potassium salt of benzyl penicillin to 6-APA, after incubation for 24 h, was 35% and 26%, respectively (**Fig. 2**). The maximum conversion rate was achieved with 3% potassium salt of benzyl

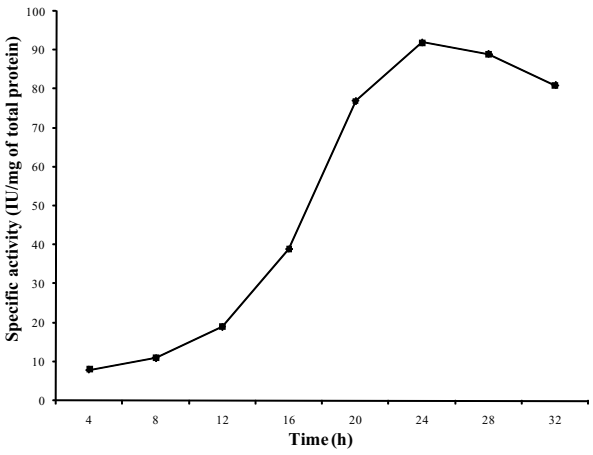

Fig. 1. Effect of time course on penicillin G acylase (PGA) activity of *Aspergillus niger* (17-M).

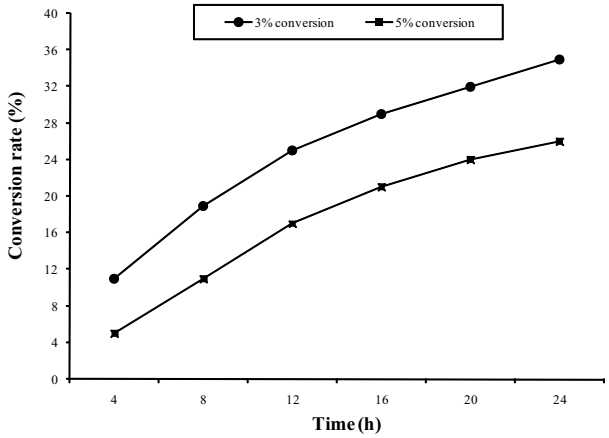

Fig. 2. Conversion curve of potassium salt of benzyl penicillin to 6-amino penicillanic acid (6-APA) by using penicillin G acylase (PGA) produced by *Aspergillus niger* (17-M).

penicillin. The hydrolysis of potassium salt of benzyl penicillin in the presence of mycelial suspension of *A. niger* (17-M) was inhibited by high concentration of substrate (5%), non-competitively by 6-APA and competitively by phenyl acetic acid (PAA), which are the products of hydrolysis reaction. The steady state kinetic of forward deacylation reaction of potassium salt of benzyl penicillin in the presence of PGA induces one substrate and two products. This activity of the enzyme was considered sufficient for practical production of 6-APA. Xian and Wang[25] reported the hydrolysis conversion of potassium salt of benzyl penicillin in the range of 2.5-12.5%.

Separation and purification of 6-APA produced by A. niger. During separation and purification of 6-APA from the reaction mixture produced by mycelial suspension of *A. niger* (2.0 g potassium benzyl penicillin in 250 ml solution, catalyzed by PGA from 800 ml of fermentation broth), 0.18 g of 6-APA was obtained with 30% conversion and 18% purification yield. 6-APA was then analyzed on thin-layer chromatographic plate to test and compare with standard 6-APA.

Kinetic assessment of enzyme hydrolysis. Km value of 0.82 mM and 1/Vmax of 0.6553 micromole minute^{-1} (Standard error, 0.001) was obtained from *A. niger* (17-M). The Michaelis constant (Km) indicated better affinity of the enzyme penicillin G acylase (PGA) produced by the strain of *A. niger* 17-M towards the substrate (**Fig. 3**).

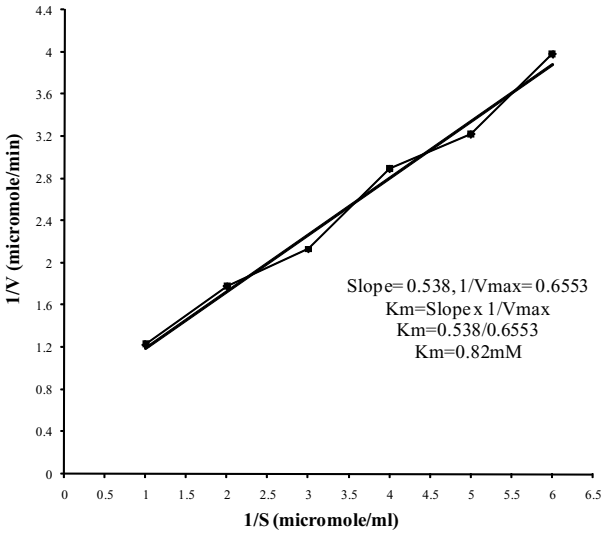

Fig. 3. Kinetic assessment of enzyme hydrolysis by *Aspergillus niger* (17-M).

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LITERATURE CITED

1. Arroyo, M., I. de la Mata, C. Acebal and M. P. Castillón. 2003. Biotechnological applications of penicillin acylases: state of the art. *Appl. Microbiol. Biotechnol.* 60: 507-514.
2. Cheng, S., D. Wei., Q. Song and X. Zhao. 2006. Immobilization of permeabilized whole cell penicillin G acylase from *Alcaligenes faecalis* using pore matrix crosslinked with glutaraldehyde. *Biotechnol. Letters* 28: 1129-1133.
3. Collins, C. H. and M. Layne. 1990. *Microbiological Methods*. Butterworths, London. 409 pp.
4. Frazier, W. C. and D. C. Westhoff. 1988. *Food Microbiology*. McGraw-Hill Publishing Company, New Dehli. 540 pp.
5. Garcia, A. M. and G. S. Fernandez. 1984. Use of different culturing techniques. *J. Food. Protection* 47: 629-635.
6. Grafkova, J. and L. Sobotkova. 2002. Penicillin G acylase-synthesis, regulation, production. *Cesk. Slov. Farm*. (Czech Medical Association Journal) 51: 6-10.
7. Koneman, E. W. and G. D. Roberts. 1985. *Practical Laboratory Mycology: Dermatophyte Identification Scheme*. Williams and Wilkins. Baltimore. 350 pp.
8. Lindsay, J. P., D. S. Clark and J. S. Dordick. 2004. Combinatorial formulation of biocatalyst preparations for increased activity in organic solvents: salt activation of penicillin amidase. *Biotechnol. Bioengg*. 85: 553-560.
9. Liu, J., X. Yang and J. Wu. 1989. A rapid method for screening high activity penicillin acylase producing strains. *Weishengwuxue Tongbao*. 16: 298-299.
10. Masarová, J., D. Mislovicová, P. Gemeiner and E. Michalková. 2001. Stability enhancement of *Escherichia coli* penicillin G acylase by glycosylation with yeast mannan. *Biotechnol. Appl. Biochem.* 34: 127-133.
11. Nam, D. H. and D. D. Ryu. 1979a. Biochemical properties of penicillin amidohydrolase from *Micrococcus luteus*. *Appl. Environ. Microbiol*. 38: 35-38.
12. Nam, D. H. and D. D. Ryu. 1979b. Preparation of 6-APA using penicillin amidohydrolase from *Micrococcus luteus*. *J. Ferment. Technol.* 57: 141-146.
13. Nasira, B., S. Quratulain and M. A. Kashmiri. 2004. Shake flask studies for the production of penicillin amidohydrolase from fungi. *Pak. J. of Sci. Res*. 56: 117-120.
14. Okachi, R., I. Kawamoto, M. Yamamoto, S. Takassawa and T. Nara. 1993. Isolation of penicillinase-deficient mutants of *Kluyvera citrophila* K3641. *Agric. Biol. Chem.* 37: 335–339.
15. Pinotti, L. M., R. G. Silva., R. C. Giordano and R. L. C. Giordano. 2002. Inoculum studies in production of penicillin G acylase by *Bacillus megaterium* ATCC 14945. *Appl. Biochem. Biotechnol*. 98-100: 679-686.
16. Plhacková, K., S. Becka., F. Skrob and P. Kyslik. 2003. Isolation and characterization of a new strain of *Achromobacter* sp. with β-lactam antibiotic acylase activity. *Appl. Microbiol. Biotechnol*. 62: 507-516.
17. Polizzi, K. M., J. F. Chaparro-Riggers, E. Vazquez-Figueroa and A. S. Bommarius. 2006. Structure-guided consensus approach to create more thermostable penicillin G acylase. *Biotechnol. J.* 1: 531-536.
18. Quratulain, S., B. Nasira and M. A. Kashmiri. 2006. Development and characterization of a potent producer of penicillin G amidase by mutagenization. *World J. Microbiol. Biotechnol*. 22: 213-218.
19. Quratulain, S., B. Nasira and M. A. Kashmiri. 2006. Characterization of amidohydrolytic activity of *Bacillus megaterium* in submerged fermentation. *Pak. J. Sci. Ind. Res.* 48: 52-54.
20. Roshan, J. I. and K. Ganapathi. 1960. Carbohydrate constituents of *Penicillium chrysogenum* grown in media with different sources of carbon. *J. Sci. Ind. Res*. 19: 207-222.
21. Senthivel, S. G. and J. S. Pai. 1998. Fermentative production of penicillin acylase. *J. Sci. Ind. Res*. 57: 781-784.
22. Shewale, J. G. and H. Sivaraman. 1989. Penicillin acylase: enzyme production and its application in the manufacture of 6-APA. *Process Biochem*. 24: 146-154.
23. Steel, R. G. D., D. Dickey and J. H. Torrie. 1996. *Principles and Procedures of Statistics, A Biometrical Approach*. McGraw-Hill Publishing Company, London. 225 pp.
24. Torres-Bacete, J., M. Arroyo, R. Torres-Guzmán, I. de la Mata, M. P. Castillón and C. Acebal. 2000. Optimization of 6-aminopenicillanic acid (6-APA) production by using a new immobilized penicillin acylase. *Biotechnol. Appl. Biochem*. 32: 173-177.
25. Xian, H. and Z. Wang. 2001. Immobilization of penicillin G acylase on polyacrylonitrile fiber. *Wei Sheng Wu Xue Bao* 41: 475-480.
26. Yang, S., H. Huang, R. Zhang, X. Huang, S. Li and Z. Yuan. 2001. Expression and purification of extracellular penicillin G acylase in *Bacillus subtilis*. *Protein Expression and Purification* 21: 60-64.