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Full Length Research Paper

Producing penicillin V acylase from novel soil actinomycete: Identification of isolate and optimization of physico-chemical parameters

***Ashoka G. Subbulakshmi, Sunil B. L. and Bankim H. Chandrasekhar**

Division of Biochemical Sciences, National Chemical Laboratory, Pune 411008, India

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Penicillin V acylase (PVA) (EC. 3.5.1.11) is a commercially important enzyme. It plays a crucial role in the production of 6-aminopenicillanic acid (6-APA), a key intermediate in manufacture of semi synthetic β-lactum antibiotics. The present paper reports screening, isolation and identification of mesophilic soil actinomycete which produces PVA intracellularly. Optimization of fermentation conditions was carried out for the maximal production of this PVA. After 16S rRNA sequencing and subsequent phylogenetic analysis the isolate was identified as *Streptomyces* sp. AAP 1846 which is a novel species with respect to PVA production. The aim of optimization studies was to design a simple and economical fermentation medium for PVA production. Highest PVA production was observed in basal medium with soyabean and tryptone as nitrogen source and lactose in the form of whey as carbon source. Maximum PVA production was observed after 168 h at pH 6.5 in 100 ml medium in a 250 ml Erlenmeyer flask at 28°C. Optimization studies resulted in about 6-fold enhancement (387.66 IU/L) in overall enzyme activity.

Key words: Penicillin V acylase, *Streptomyces* sp., intracellular enzyme, whey, lactose.

INTRODUCTION

Since its discovery in 1928 penicillin has been the most widely used antibiotic and a preferred choice of treatment for majority of the diseases. The production of penicillin and cephalosporin antibiotics is a multi dollar industrial operation (Spence and Ramsden, 2007). However, with increasing indiscriminate use of antibiotics, pathogens have developed resistance to both penicillin and many of its commonly used derivatives. Modifying the original penicillin molecule to form newer molecules can be done by the addition of a different acyl side chain to an existing penicillin nucleus (Shewale and SivaRaman, 1989; Shewale et al., 1990) The ability of penicillin acylases to hydrolyze its substrate by specifically attacking the amide bond of the side chain, while simultaneously protecting the β-lactam amide bond, makes it an enzyme of industrial importance for production of key drug intermediates in manufacture of semi synthetic penicillin (Bruggink et al., 1998).

Penicillin acylases are members of N-terminal nucleophile (Ntn)-hydrolase superfamily. Enzymes of this superfamily show a high degree of structural similarity and have a serine, cysteine or threonine residue at the n-terminal end that accounts for their catalytic activity (Brannigan and Dogson, 1995; Onionen and Rouvinen, 2000).

Bacteria, yeast and filamentous fungi have all been implicated in production of Penicillin acylase (Arroyo et

*Corresponding author. E-mail: Ashoka.gani@hotmail.com

al., 2003). Penicillin V acylase (PVA) is primarily an intracellular enzyme and has been reported in many bacteria, fungi and yeast (Kumar et al., 2008; Pundle and SivaRaman, 1994; Pundle and SivaRaman, 1997; Sundar et al., 2011; Vandamme and Voets, 2005). Extracellular synthesis has been observed in case of prokaryotes like *Streptomyces lavendulae* (Torres Guzman et al., 1995), *Streptoverticillium* sp., and eukaryotes like *Fusarium* sp. SKF 235 and *Pleurotus ostreatus* (Shewale and Sudhakaran, 1995).

This paper reports PVA producing capability of an isolate obtained from sub surface soil from an area rich in plant detritus located 300 m above sea level near Tamini ghat (18°23'41"N 73°23'1"E) and designated as Isolate 13.

The study focuses on optimization of nutritional requirements and culture conditions to maximize production of intracellular PVA by this isolate, under shake flask conditions. Whey (4% v/v) was used as the carbon source, thus making the process economically appealing. 16S rRNA sequencing identified the isolate as a *Streptomyces* but totally unrelated to previously reported species of *Streptomyces*, capable of producing PVA. Thus, we claim this isolate to be novel with respect to PVA production.

MATERIALS AND METHODS

Materials

Media components were procured from Hi Media, India. PDAB was procured from Qualigens, India. Penicillin V Potassium Salt used in the assay was a kind gift from KDL Biotech, Khopoli, India.

Screening of penicillin V acylase producers

Bioassay for the screening of isolates in order to determine potential PVA producer was carried out using an indicator organism *Serratia marscesens* ATCC 27117 according to method developed by Meevootisom et al. (1983).

Enzyme assay

Enzyme activity of the PVA positive isolates were determined by using a colorimetric assay described by Bombstein and Evans (1965) which determines the amount of 6- Aminopenicillanic acid (6-APA) formed during the enzyme reaction. In brief, the assay was performed using 35 mg (wet weight) of cells which was obtained by centrifuging 168 h old broth culture of the organism at 10000 rpm for 10 min.

The pellet was resuspended in 1 ml of 50 mM phosphate buffer pH 6.8 containing 20 mg/ml of Penicillin V and was incubated at 40°C for 10 min. Reaction was stopped by adding citrate phosphate buffer pH 2.5. Whitish precipitate formed after addition of CPB was removed by centrifugation and the cell free supernatant was checked for presence of 6-APA. 6-APA formed was estimated using p-dimethyl amino benzaldehyde. The 6-amino group of 6-APA, reacts with p-dimethylaminobenzaldehyde (PDAB) to form a coloured Schiff's base which is estimated spectrophotometrically at 415 nm. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µmol 6-APA per minute under assay conditions.

Identification of culture by 16S rRNA sequencing

DNA isolation

DNA was isolated according to the modified method reported by Magarvey et al. (2004). In brief, Isolate 13 was grown to saturation in trypticase soy broth. The cell mass was pelleted down by centrifugation washed with 0.8% NaCl solution. The pellet was then re-suspended in TE (pH 8.0) and was lysed using a mixture of SDS and Lysozyme at RT.

Proteinase K was added to the pellet and incubated for 2 h at 37°C. Polysaccharides were removed by adding CTAB/NaCl solution followed by incubation at 65^{O}C for 10 min. The DNA was extracted from aqueous phase after wash with phenol: chloroform: isoamyl alcohol (25:24:1) and subsequently treated with chloroform: isoamyl alcohol (24:1). The aqueous phase was treated with 0.6 volumes of isopropanol and incubated at -20°C for an hour. The precipitated DNA was pelleted down and washed with 70% ethanol, air dried and dissolved in TE buffer (pH 8.0).

DNA amplification and sequencing

The 16S rRNA gene was amplified from extracted genomic DNA using universal primers 8F and 1525R in a PCR reaction mixture containing 2.5U of AmpliTaq Gold (Invitrogen) and PCR conditions were as suggested by Tajima et al. (2001) with slight modifications. The reaction was subjected to an initial denaturation at 94°C for 5min with 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min and extension at 72°C for 1 min with additional extension at 72°C for 10 min and finally held at 20°C.

Amplification was confirmed by agarose gel electrophoresis. The amplicons were then subjected to PCR clean up and sequenced using internal primers. Chromas Pro version 1.34 (Technelysium Pty Ltd., Tewantin, Queensland, Australia) was used for assembling the reads into a single contig.

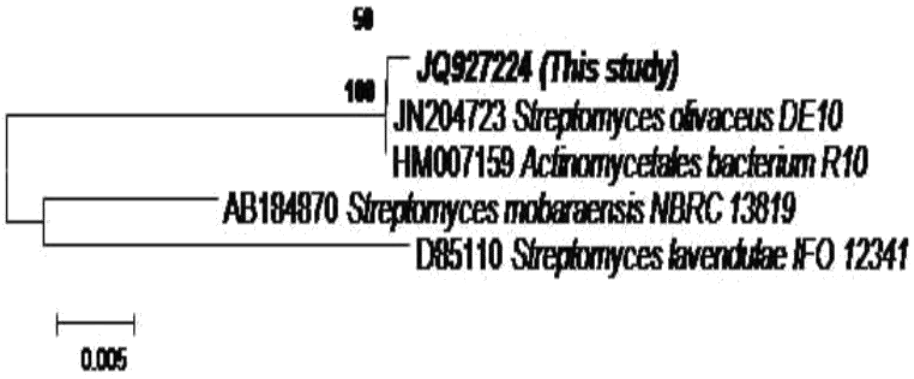

Figure 1. Unrooted phylogenetic tree obtained from 16S rRNA gene sequence of isolate 13.The neighbor joining tree shows that isolate 13 is related to *Streptomyces olivaceus* DE10.

Nucleotide sequence submission and phylogenetic analysis

The sequence obtained during this study was deposited in the GenBank database under the accession number JQ927224 (Benson et al., 2013). The sequence was subjected to database matching in NCBI BLASTn (Altschul et al., 1990). Similar sequences were subjected to multiple sequence alignment using CLUSTAL W (Thompson et al., 1994). Phylogenetic tree was computed in MEGA 4 using the neighbor joining method (Tamura et al., 2007).

Fermentation parameters

Different simple and complex carbohydrates including lactose, mannitol, mannose, glucose, fructose, cellobiose, glycerol and galactose were used as carbon sources at a concentration ranging from 1 to 3% (w/v)

.Simple and complex organic nitrogen sources comprising of urea, tryptone, peptone, yeast extract, soyabean meal, were used at concentrations ranging from 0.2 to 1.0%. Inorganic nitrogen sources consisting of diammonium phosphate, ammonium sulphate, ammonium nitrate, ammonium dihydrogen phosphate, ammonium chloride, ammonium molybdate, sodium nitrate and ammonium acetate were used at concentrations 0.2, 0.5 and 1.0% (w/v). The media used for testing the above variables was Gause's No. 1 minimal media (Labeda and Shearer, 1990) from which starch was replaced by the selected carbon source. The optima of carbon and nitrogen sources obtained were preserved in the medium used for optimizing growth pH, growth temperature, and aeration. 10% seed culture was used and experiments were conducted thrice for the sake of reproducibility.

An optimum temperature condition for maximum enzyme production was determined by growing the culture under most favorable nutritional conditions as previously determined. The 250 ml Erlenmeyer flasks containing 100 ml of optimized medium were incubated at 25, 28, 37 and 40, 45 and 50°C under shake flask conditions (180 rpm) for 168 hours respectively. Optimum pH was determined by subjecting the cells to pH ranging from 6.5 to 8.5, at an optimum temperature of 28°C. To study the effect of aeration upon enzyme productivity, the volume of the optimized media used for fermentation was varied from 25 to 150 ml with 25 ml increments in a 250 ml Erlenmeyer flask, under optimum conditions of temperature and pH.

RESULTS

Qualitative screening to identify potential PVA producers

Isolates obtained from soil were screened for their potential to produce PVA. When subjected to screening using an indicator strain, one of the isolates was found to inhibit growth of the test organism it was designated as isolate 13 and was used for further studies.

Identification of PVA producer

The 16S rRNA gene of Isolate 13 was sequenced and a 1469 bp sequence was obtained which has been deposited under GenBank accession number JQ927224. The isolate was designated as *Streptomyces* sp. AAP 1846 . The analysis of the gene sequence revealed that it grouped in an unrooted phylogenetic tree with *Streptomyces olivaceus* DE10, which is previously unreported for PVA production. Additionally, in the phylogenetic tree, we also included two more sequences of organisms belonging to the genus *Streptomyces* which were reported to produce PVA in order to verify evolutionary distance and uniqueness of our isolate (Figure 1).

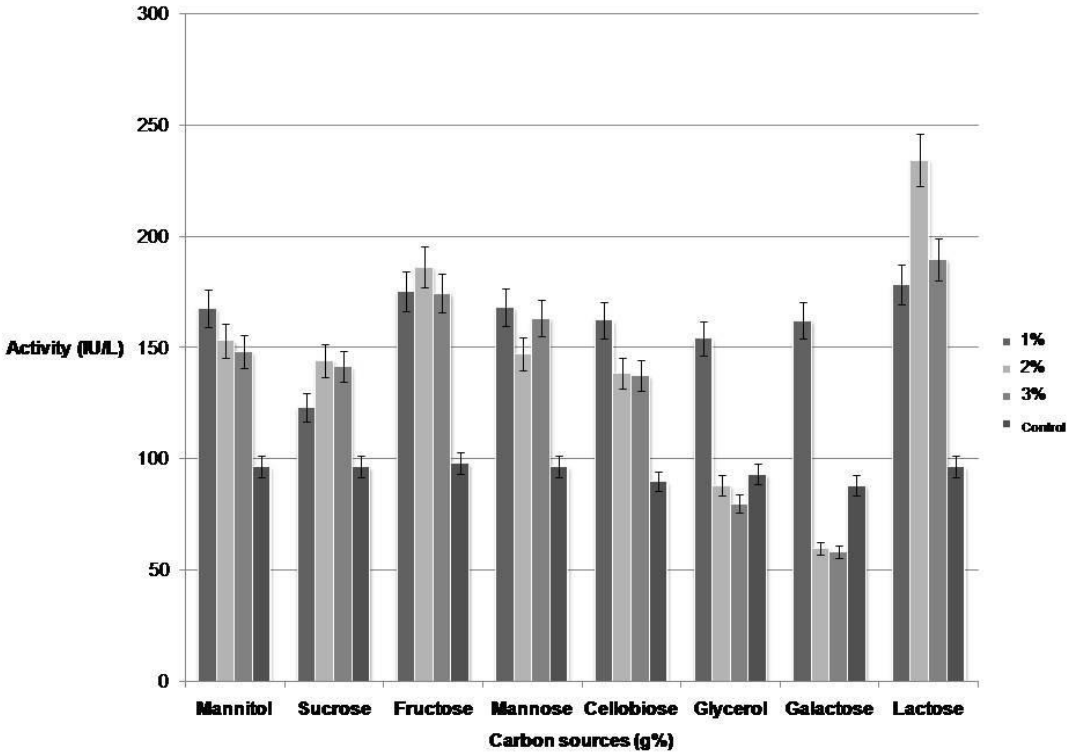

Figure 2a. Effect of carbon sources on enzyme activity at concentrations 1, 2 and 3%, compared to control.

Effect of carbohydrates on PVA production

Torres-Bacete et al. (2005) studied the effect of lactose, glucose and glycerol on PVA production and found that all the carbon sources showed catabolite repression at 1% concentration. On the contrary, in the present study, a slight increase in enzyme activity with many different sugars at 1% concentration was observed. From Figure 2a, it is evident that lactose at a concentration of both 1 and 2% showed higher activity (1.84 and 3.10 fold respectively) when compared to the other carbon sources and the control. However when the concentration of lactose was increased to 3%, it was inhibitory to enzyme activity (Figure2c). Hence, 2% lactose (w/v) was the chosen carbon source for further studies.

Whey obtained from cheese making industry was treated as the original concentrated sample from which aliquots were withdrawn to make the final media concentration such as 1, 2, 3, 4 and 5% (v/v) (Figure 2b). Use of 4% (v/v) whey as a source of lactose showed a 2 fold increase in enzyme activity, when compared to commercially available lactose which was being used at a concentration of 2% w/v (Figure 2d). Any further increase in the concentration of whey, the enzyme activity remained unchanged. Though there have been reports of lactose being used as a carbon source, this is the first report of whey being used as a carbon source to enhance PVA productivity.

Effect of inorganic nitrogen sources on enzyme activity

Inorganic nitrogen sources were used at concentrations of 0.2, 0.5 and 1.0% (w/v). Diammonium phosphate showed maximum activity at 0.2% (w/v) when compared with the other inorganic nitrogen sources. At all concentrations, sodium nitrate, ammonium acetate, ammonium dihydrogen phosphate, ammonium chloride and ammonium nitrate did not show significant activity when compared to the control. Ammonium molybdate showed complete growth inhibition even at the lowest concentration of 0.2%. Figure 3a shows PVA activity determined in the presence of diammonium phosphate and ammonium sulphate when added to the basal medium respectively.

Effect of organic nitrogen sources on enzyme activity

The Figure 3b is indicative of the fact that tryptone at a concentration of 0.2% (w/v) showed maximum activity; followed by soyabean meal, when compared to other organic nitrogen sources. Moreover when tryptone and

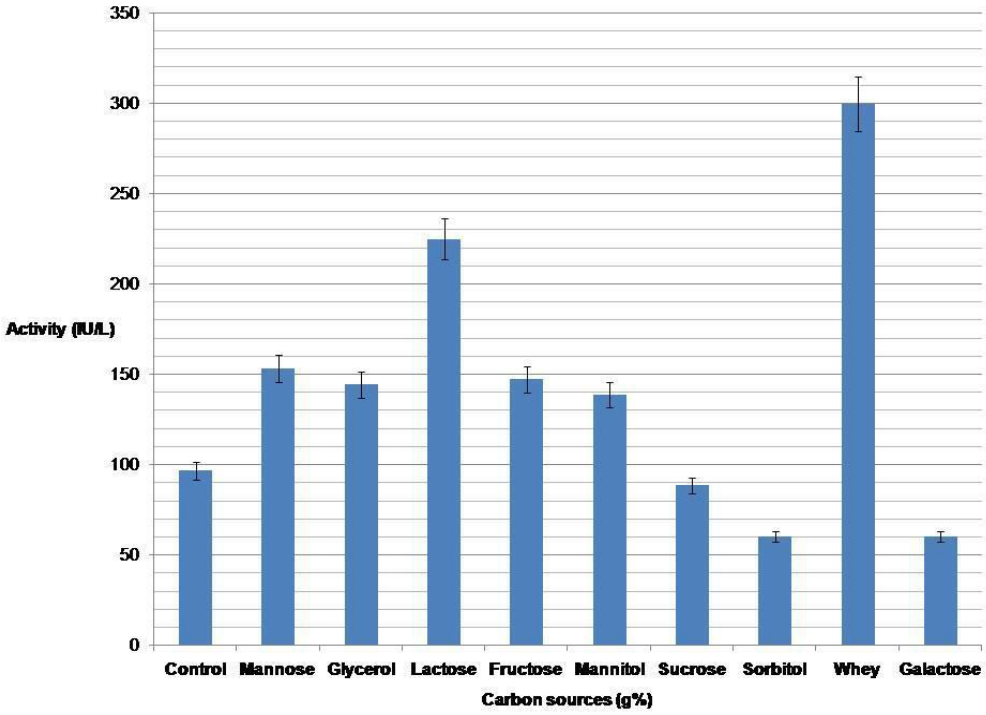

Figure 2b. Effect of addition of whey (2%) in the medium on enzyme activity.

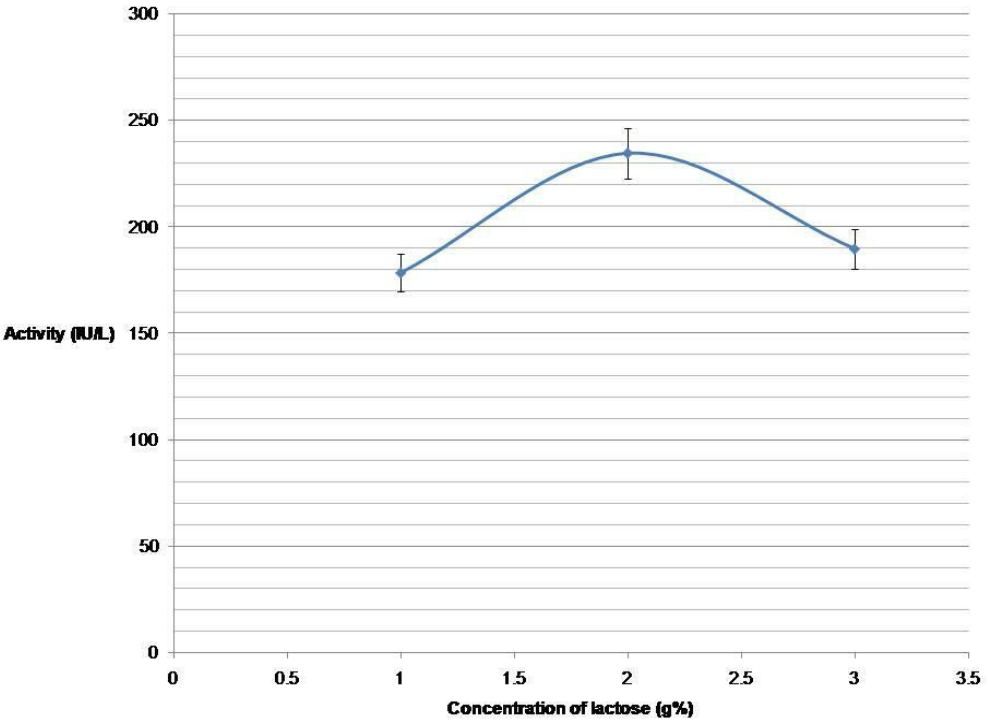

Figure 2c. Effect of lactose at concentrations 1, 2 and 3% on enzyme activity.

soyabean meal were used together, enzyme activity enhanced two-fold compared to when either of them were used individually as a nitrogen source in basal medium. However, to make the process cost-effective, tryptone (expensive source) can be replaced with a relatively inexpensive soya - bean meal since both show same

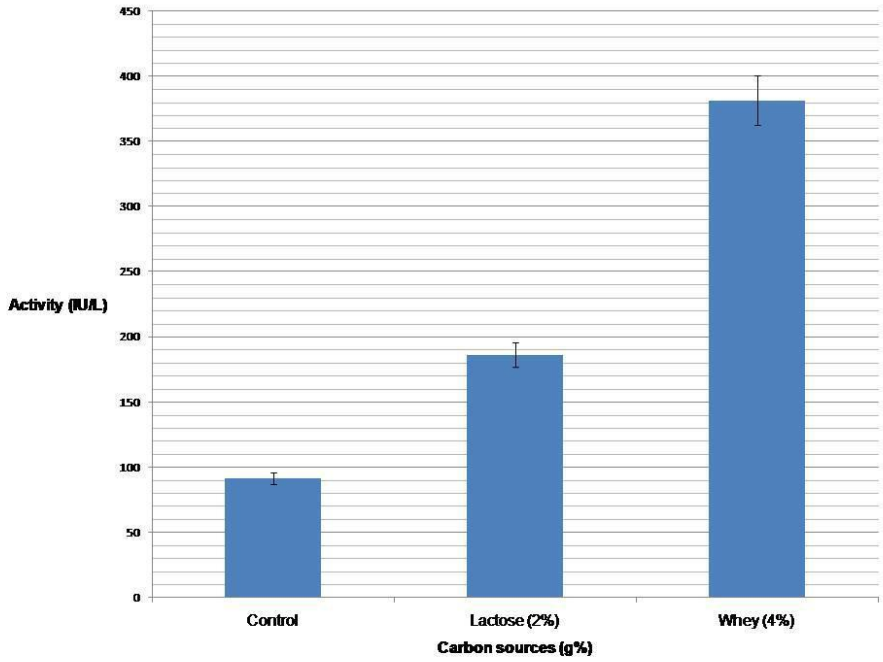

Figure 2d. Comparison of the effect of lactose and whey on enzyme activity.

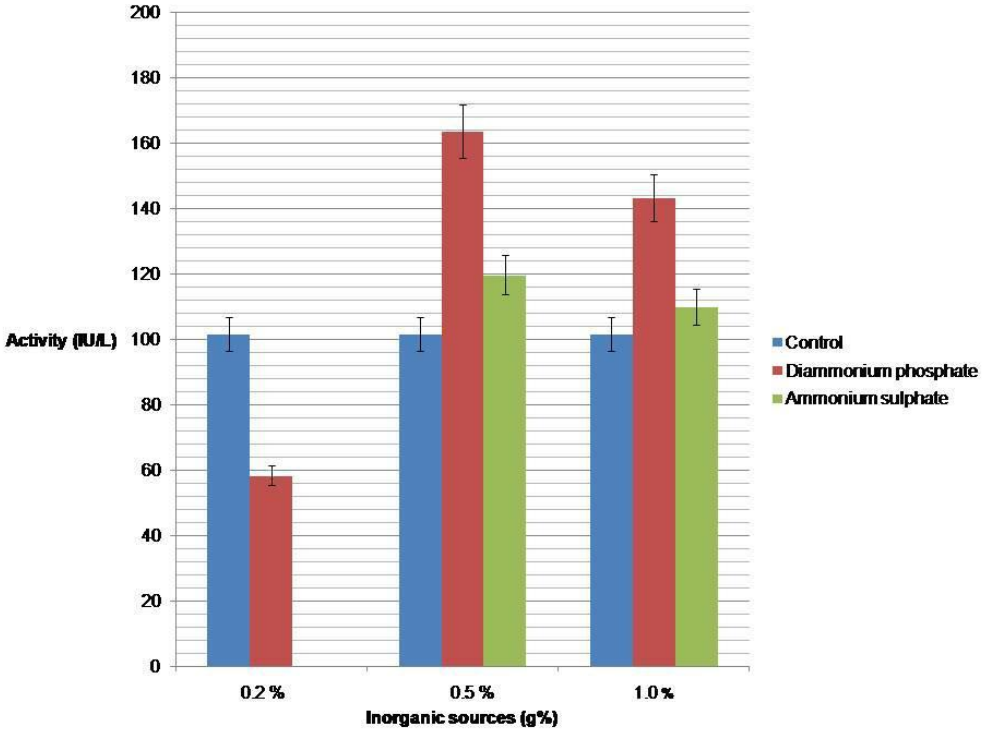

Figure 3a. Effect of inorganic nitrogen sources on enzyme activity.

activity (Figure 3c). In case of *Streptomyces* sp. AAP1846, with the addition of yeast extract in the growth medium PVA production did increase marginally but the enzyme was still produced intracellularly.

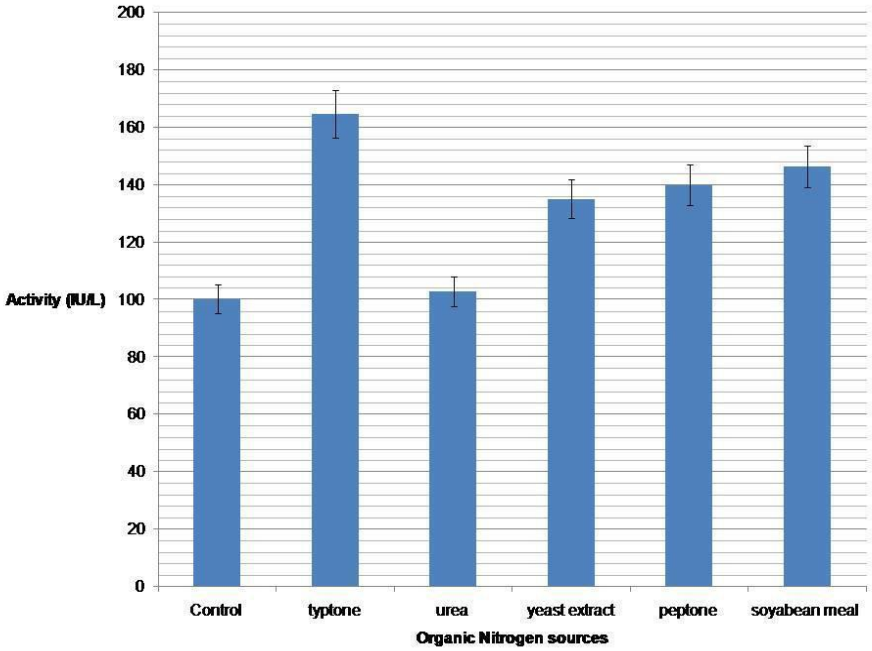

Figure 3b. Effect of organic nitrogen sources on enzyme activity.

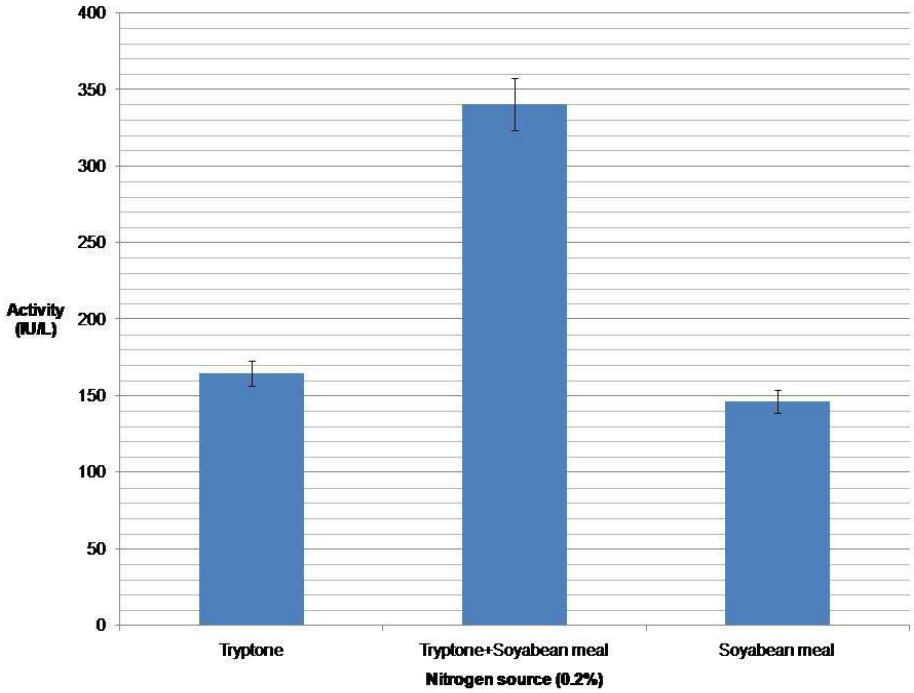

Figure 3c. Comparison between effect of tryptone and soyabean meal, when used alone and in combination, on enzyme activity.

Effect of aeration on enzyme activity

Various volumes of 25 to 150 ml were dispensed in 250 ml Erlenmeyer flasks to study the effect the aeration on enzyme activity. Maximum enzyme activity was observed at a volume of 100 ml indicating that micro-aerophillic

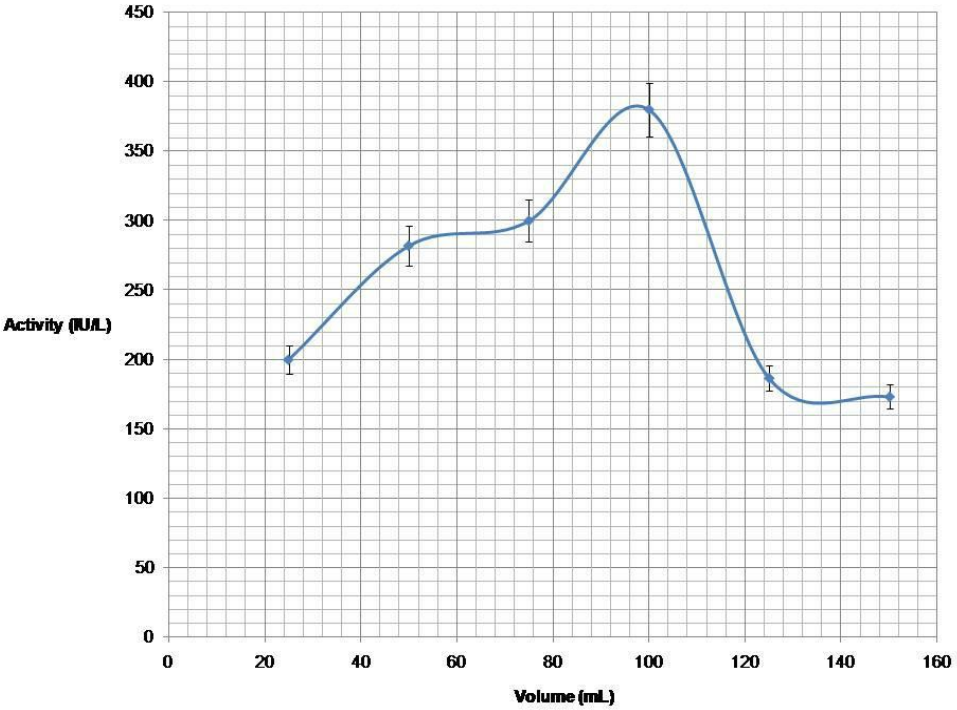

Figure 4. Effect of aeration on intracellular enzyme activity.

conditions were preferred for PVA production by *Streptomyces* sp. AAP1846 (Figure 4). Hence, this volume of medium was used for further studies. A subsequent increase in volume beyond 100 mL proved to be detrimental to PVA synthesis due to decreased dissolved oxygen content in the flask. Torres et al. (2005) also reported that volume of medium/volume of flask ratio of 0.4 (that is, 100 ml in case of 250 ml Erlenmeyer flask) stimulated PVA production.

Effect of growth medium pH on enzyme activity

Streptomyces sp. AAP 1846 produced PVA that has an optimum growth pH of 6.5 (Figure 5), which is typical of the reported pH range for PVA production from microbial sources (Spence and Ramsden, 2007). A further increase in pH of the growth medium to pH 7.0 showed a significant drop in the enzyme activity, beyond which, no appreciable change was seen.

Effect of growth temperature on enzyme activity

Maximum activity was obtained at an incubation temperature of 28°C for *Streptomyces* sp. AAP 1846. Further increases in temperature to 37°C lead to a substantial decrease in growth of cells as well as in enzyme activity; whereas, nil activity and no growth was observed at an incubation temperature of 50°C. Thus *Streptomyces* sp. AAP 1846 is a mesophilic PVA producer (Figure 6).

DISCUSSION

The production of 6-APA remains, undisputedly, of prime importance in the development of semi-synthetic penicillins. The chemical process of 6-APA production has been largely replaced by the enzymatic process, which necessitates work in exploring newer and better sources for penicillin acylases. PVA has been shown to possess superior characteristics that are suitable for the fermentation processes, but much work is needed to successfully exploit this enzyme. PVA from *Streptomyces lavendulae* has been worked upon extensively. This has triggered interest in actinomycetes as a source of PVA as the current area of research.

Till date, there are reports of only four species of actinomycetes, capable of producing PA at the commercial level. Isolation and identification of newer actinomycetes is of equal importance as is optimization of physicochemical parameters.

The culture medium and conditions were optimized for penicillin acylase production by *Streptomyces* sp. AAP1846. Reported carbon sources include sucrose, lactose, galactose, sorbose, glycerol, sorbitol, inositol, mannitol, corn starch, soluble starch and cellulose (Sudhakaran and Shewale, 1993). Carbon sources tried in the study include mannose, maltose, glycerol, lactose,

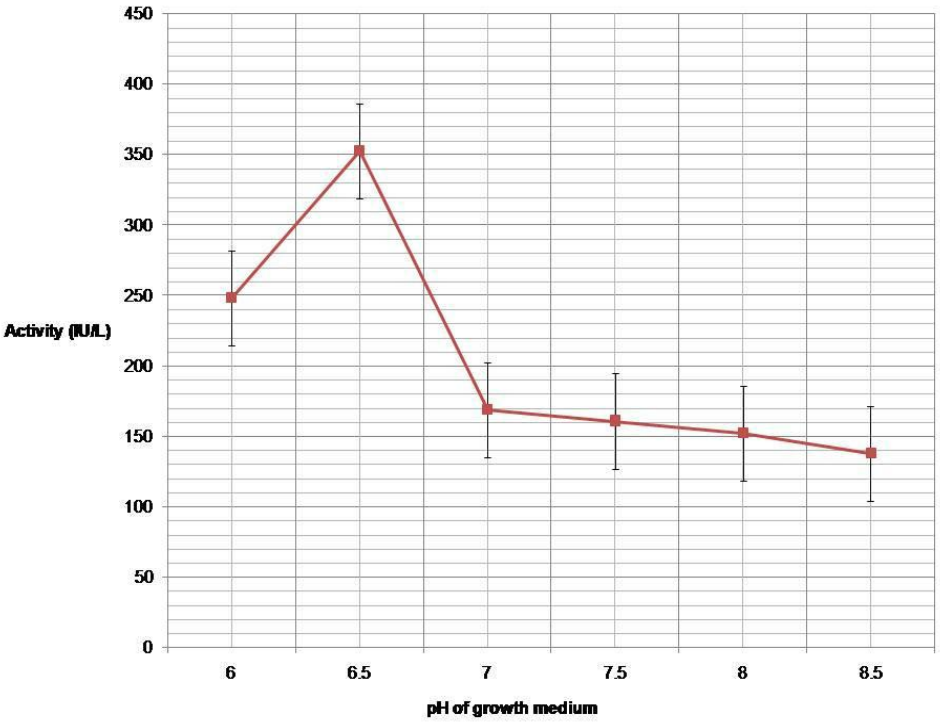

Figure 5. Effect of media pH on intracellular enzyme activity.

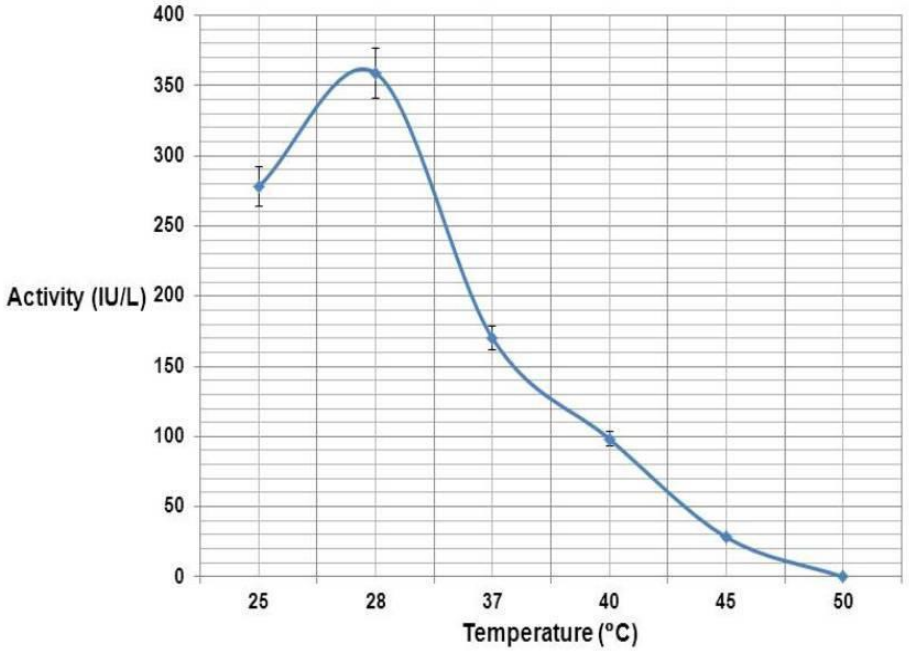

Figure 6. Effect of growth temperature on intracellular enzyme activity.

fructose, mannitol, sucrose and sorbitol. Out of these carbohydrates, lactose when used as carbon source, showed maximum enzyme activity. The ultimate application lies in the production of PVA from *Streptomyces* sp. AAP 1846 by fermentation at a large scale. One of the factors for lucrative production of the enzyme is the use of

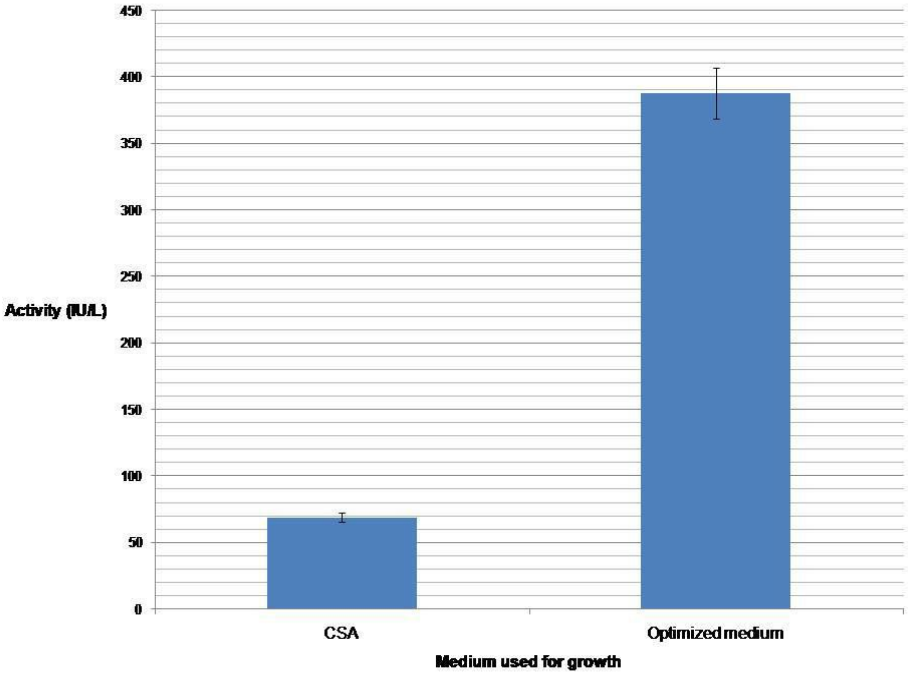

Figure 7. Comparison of enzyme activity in optimized and routine culture medium.

inexpensive nutrient sources. Thus, whey, a by-product of the cheese-making industry, was used in the medium instead of commercially available lactose powder and enzyme activity was estimated.

Sudhakaran and Shewale (1993) had reported incorporation of several inorganic nitrogen sources in the basal medium at concentrations ranging from 0.1 to 0.5% (w/v) while studying PVA production in *Fusarium* sp. In their study, ammonium phosphate showed a higher enzyme activity as compared to diammonium phosphate; in the above specified range. However, there are no reports on effect of inorganic nitrogen sources on enzyme production in case of *Streptomyces* sp., and is being explored here for the first time.

Use of complex organic nitrogen sources, like tryptone and soya bean meal (which were eventually became a part of the optimized medium in this study) was supported by previous reports that claimed organic nitrogen sources enhanced penicillin acylase production. Complex organic nitrogen sources indeed enhance the production of PVA according to Shewale and Sudhakaran (1997).

Shewale and SivaRaman (1989) reported that yeast extract and peptone gave a higher yield than tryptone. However, our results indicate that tryptone gave a higher enzyme activity than yeast extract and peptone. Also worth noticing is the enhanced production of the enzyme when soyabean meal is incorporated in to the medium. Additionally, it has been reported by Torres-Bacete et al. (2005) that yeast extract enhanced both, production and extracellular secretion of PVA. Another report by Chauhan et al. (1998) also accounts for the use of soyabean meal, groundnut cake and corn-steep liquor as complex organic nitrogen sources used to enhance PVA production by *Chainia* sp.

However the overall exercise of media optimization for penicillin V acylase production in this study using whey showed approximately 6 fold (387.66 IU/L) increase in enzyme activity which is very significant (Figure 7).

Conclusion

The conditions that supported maximum production of intracellular PVA from *Streptomyces* sp. AAP 1846 were optimized and there was found to be a six fold increase in enzyme production. This isolate was found to be completely novel with respect to PVA production since it did not clade with any of the previously reported PVA producing actinomycetes. Use of renewable carbon source, 4% (v/v) whey, in the optimized medium makes this process an appealing candidate from industrial scale up perspective. Optimum pH of the growth medium was found to be 6.5, which is comparable to previous reports. Further studies on enzyme purification and characterization from this novel soil actinomycete are underway.

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