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Penicillin Acylase Production By *Micrococcus luteus* and *Staphylococcus* spp. Isolated from Soda Lake.

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ABSTRACT
Two strains of *M. luteus* and a *Staphylococcus* spp capable of producing penicillin acylse were isolated from lonar lake water. Optimum penicillin acylse production time for *M. luteus* was 72hrs and 48hrs for *Staphyloccus* spp. Elevated temperature (40 to 50°C) better suited penicillin acylse production. Glucose and sucrose stimulated penicillin acylse production by *M. luteus* and *Staphyloccus* spp respectively. Penicillin acylse from *M. luteus* worked optimally at neutral to slightly alkaline pH and within 60 to 80°C. *Staphylococcus* spp. worked better at pH 6. Penicillin acylse was more active against ampicillin.

Keywords: Ampicillin, Lonar lake, *M. luteus*, Penicillin acylase, *Staphylococcus.*

1. INTRODUCTION
Enzymatic method for large scale production of 6-aminopenicillanic acid employs penicillin acylase or penicillin amidohydrolase (EC 3.5.1.11) thus is one of the most important enzyme applied in the pharmaceutical industry [1]. 6-aminopenicillanic acid is starting material for synthesis of semisynthetic penicillin [2]. Many genera of molds, yeast and bacteria produce penicillin acylases. Among them, enzyme produced by *E. coli* is the most well-characterized and common one for industrial application. *E. coli* is known to produce an intracellular penicillin acylase that can be induced by phenylacetic acid [3].
Due to high industrial importance of penicillin acylase, numerous efforts have been made towards screening for strains overproducing this enzyme [4]. Penicillin acylase is, in general, produced in fermentative process and is obtained from either mutated or natural variant strains [5]. The catalytically active enzyme derived from *E. coli* is in the form of α, β heterodimer localized in periplasmic space of cell [6]. Penicillin acylase has also been used for synthesis of amoxicillin [7].
The *E. coli* penicillin acylase being intracellular is quite difficult to purify. Even if whole cell are used for catalysis it would pose substrate/product diffusion problem thereby putting break on speed of reaction. The present communication describes our findings on penicillin acylase producers *M. luteus* and *Staphylococcus* spp. isolated from Lonar Lake, a soda lake (In Buldhana district (MS), India which is an alkaline habitat. Very few reports of penicillin acylase of these two are available. Thus we have also optimized few production parameters and reaction conditions.

2. MATERIALS AND METHODS
2.1. Isolation of Penicillin G resistant bacteria: For enrichment 1.0mL lonar lake water sample was added to nutrient broth (g/L, Beef extract 3; Bacteriological peptone 5; NaCl 5) tubes with increasing pH (7, 8, 9 and 10). The broth was supplemented with 0.1% benzathine penicillin. For isolation loop full enriched sample was streaked on nutrient agar (Nutrient broth + 2% agar agar) + benzylpenicillin of respective pH.

2.2. Screening of Penicillin Acylase Producers: Three isolates LL1, LL2 and LL3 were grown for 24hrs on nutrient medium composed of g/L, $MgS0_4$ 0.2;KH_2P0_4 3.0;K_2HP0_4 7.0; Yeast extract 5.0; Peptone 20.0; Benzylpenicillin 10; Agar agar 20. Cultures were then screened for PAG activity by acidimetric method (8). The test reagent was composed of Penicillin G, 600mg + Phenol red (0.5%), 0.5mL + Sterile distilled water, 4.5 mL. The test reagent was adjusted to pH 8.5 with 1.0 M NaOH giving violet color to the solution. Three drops of freshly prepared reagent was added to 0.5mL cell suspension of test cultures. The cultures positive from penicillin acylase activity were subjected to biochemical characterization for identification [9].

2.3. Penicillin Acylase Assay: Penicillin acylase assay was done by p-Di-Methyl Amino Benzyldehyde (PDAB) method [10]. The PDAB reagent composed of PDAB 1.5g, Acetic acid 100mL, Methanol 60mL, and water 40mL. Reaction mixture composed of Cell suspension, 1mL + 0.5% Penicillin G in 0.1M Phosphate buffer, 5mL. After incubation at 40ºC for 40 min

1.5 mL PDAB reagent was added. The 6-APA produced was measured spectrophotometrically at 415 nm. One unit of penicillin acylase was taken as amount of enzyme required to liberate 1 µMol of 6-amino penicillanic acid per minute under assay conditions.

2.4. Nature of Penicillin Acylase: For determination of nature of enzyme (extracellular/ intracellular) penicillin acylase producer organism was grown in presence of Penicillin G. Cells were separated from rest of medium by centrifugation and penicillin acylase activity in pelleted cells and cell free supernatant was determined. .

2.5. Optimization of Growth Conditions for Penicillin Acylase Production: For time course and temperature optimization the production media used composed of g/L, Beef extract 3.0; Bacteriological peptone 5.0; NaCl 5.0; Benzylpenicillin 10; pH 7. For time course optimization the 5% inoculums of test organism was inoculated in production medium. Penicillin acylase activity was determined after every 24hrs for 4days. For temperature optimization inoculated production medium was incubated at different temperatures (20, 30, 40, 50 and 60ºC). Effect of glucose, lactose and sucrose on penicillin acylase production was studied. For this the production media was supplemented with 0.2% test sugar.

2.6. Characterization of Penicillin Acylase: Effect of temperature on penicillin acylase activity in range 20, 30,…80ºC was determined. To study effect of pH on penicillin activity the substrate solution was prepared in buffers with different pH (4, 5, 6, . . 10). To check specificity penicillin acylase was reacted with different beta lactum antibiotics – ampicillin, piperacillin, benzathine penicillin, Benzyl penicillin.

3. RESULT

Three isolates were obtained from plate with pH 7, 9 and 10, no growth was observed on agar plates of pH 8. Isolates were given codes as LL1, LL2 and LL3. In acidimetric test we found that all three cultures changed the color of phenol red from violet to deep red (Fig: 1) which persisted for more than 48hrs this confirms all cultures are penicillin acylase positive and the penicillin resistance is not just because of β-lactamase. Morphological appearance and biochemical activities of three bacteria are enlisted in Table 1. The results were compared with Bergey's manual of Determinative Bacteriology. From this it can be concluded that LL1 & LL3 are strains of *Micrococcus luteus* and LL2 is *Staphylococcus* spp. Two *M. luteus* were distinguished as *M. luteus* I and *M. luteus* II according to pH in which it was grown 7 and 10 respectively. Penicillin acylase activity of both strains of *M. luteus* and *Staphyloccus* spp. were showing almost double in supernatant than in pellet (Table 2). This shows that the enzyme is largely extracellular.

The penicillin acylase production conditions for all cultures were optimized. Penicillin acylase production by both *M. luteus* strains reached maximum on 3rd day of incubation whereas with *Staphyloccus* spp. the peak production was on 2nd day (Table 3). In case of optimum production temperature we found *M. luteus* I produced penicillin acylase at higher level when grown at 40°C and other two did same at 50°C (Table 4). Production medium when supplemented with glucose penicillin acylase production by *M. luteus* was stimulated, whereas sucrose stimulated penicillin acylase production by *Staphylococcus* spp (Table 5).

The penicillin acylase temperature optimum for *M. luteus* I and *Staphylococcus* spp. was found to be at 80 °C and *M. luteus* II was working best at 60 °C (Figure 2). Pencillin acylase of *M. luteus* worked better in slightly alkaline pH whereas penicillin acylase of *Staphylococcus* spp. was working best at pH 6 (Figure 3). Substrate specificity of penicillin acylase against few other beta lactum antibiotics was checked. The results are presented in histogram (Figure 4). All three penicillin acylase worked with almost similar efficiency against Penicillin G, Piperacillin and Benzathine penicillin. Whereas all three much more rapidly deacylated ampicillin.

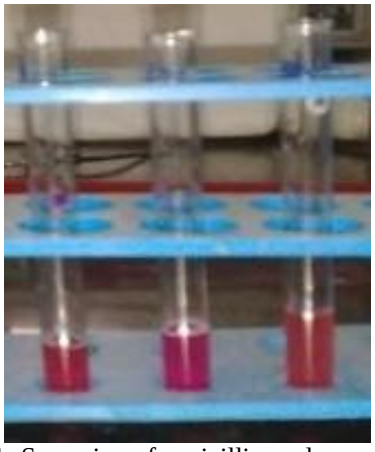

Figure 1: Screening of penicillin acylase producers.

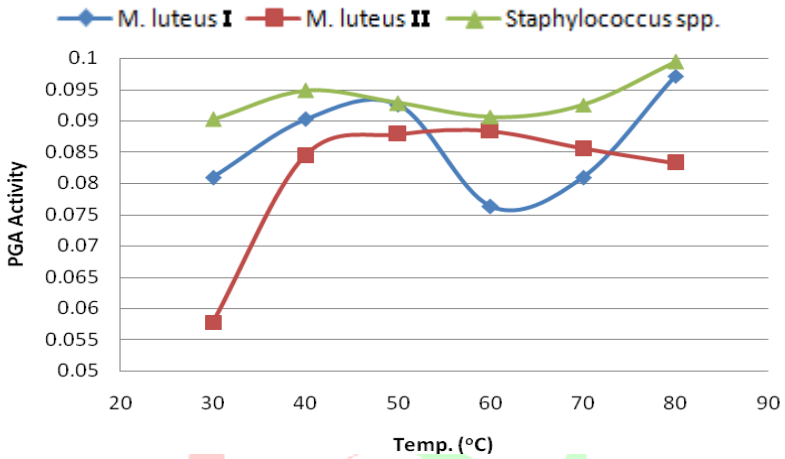

Figure 2: Effect of temperature on penicillin acylase activity.

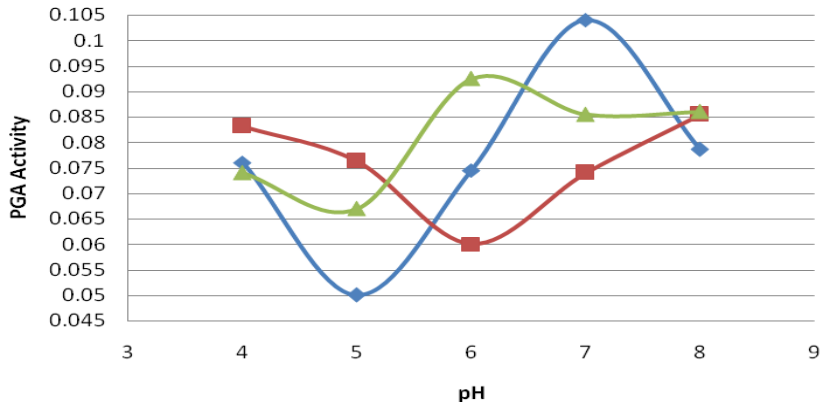

Figure 3: Effect of pH on penicillin acylase activity.

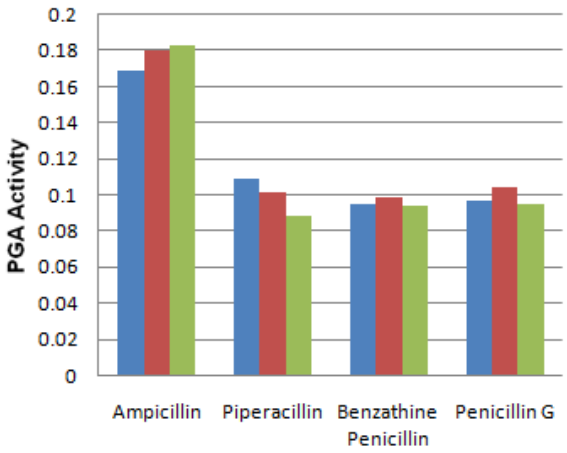

Figure 4: Substrate specificity of penicillin acylase.

Table 1: Morphological appearance and Biochemical activities of penicillin acylase producers.

Characters	**LL1**	**LL2**	**LL3**
Pigmentation	Yellow	Off white	Yellow
Shape of cells	Cocci	Cocci	Cocci
Arrangement	Chains	Clusters	Chains
Gram Nature	+ve	+ve	+ve
Motility	-ve	-ve	-ve
Indole Production	-ve	-ve	-ve
Methyl red	-ve	-ve	-ve
Voges Proskeur Test	-ve	-ve	-ve
Citrate Utilization	-ve	-ve	-ve
Glucose Fermentation	-ve	-ve	-ve
Sucrose Fermentation	-ve	+ve	+ve
Fructose Fermentation	-ve	-ve	-ve
Lactose Fermentation	-ve	-ve	-ve
Catalase Test	+ve	+ve	+ve

Table 2: Determination of nature of penicillin acylase.

Culture	**PGA activity in Supernatant**	**PGA activity in Pellet**
M. luteus I	0.0832	0.0346
Staphyloccus spp.	0.0879	0.0416
M. luteus II	0.0693	0.0300

Table 3: Time course of PGA production.

Incubation Time (hr)	***M. luteus* I**	***M. luteus* II**	***Staphylococcus* spp.**
24	0.064	0.065	0.076
48	0.074	0.0925	0.0948
72	0.1087	0.1040	0.0855
96	0.1017	0.0786	0.0902

Table 4: Temperature optimization.

Temperature (°C)	*M. luteus* I	*M. luteus* II	*Staphylococcus* spp.
20	0.0740	0.0601	0.0751
30	0.0763	0.0717	0.0717
40	0.0832	0.0693	0.0879
50	0.0602	0.0751	0.0902
60	0.0624	0.0763	0.0855

Table 5: Effect of Carbon source.

Carbon Source	*M. luteus* I	*M. luteus* II	*Staphylococcus* spp.
Glucose	0.0185	0.578	0.0092
Sucrose	0.0138	0.037	0.0393
Lactose	0.010	0.0370	0.0254

4. DISCUSSION

In present study *M. luteus* and *Staphylococcus* spp. extracellular penicillin acylase producers were isolated from lonar lake water. Few characteristics of enzymes like optimum temperature, pH, substrate specificity was determined and few production parameters were optimized. Nam and Ryu, 1979 reported that penicillin acylase of *M. luteus* showed optimum activity at 36^{o}C and 7.5 pH. Our findings are agree with the this pH optima but could tolerate higher temperature. The also reported the enzyme was active against penicillin V and ampicillin (11). Sedigheh Javadpour et al. 2002, characterized penicillin acylase of *E. coli* and the found that the pH and temperature optimum was 8 and 60^{o}C (1). According to Hesham et al. 2009, penicillin acylase production by *E. coli* reaches maximum after 19 hrs of incubation remained stable up to 24hrs after which it declined. Thus our isolates were slower compared to *E. coli* (6). Szentirmai, 1964, proposed the inhibitory effect of carbohydrates on penicillin acylse production when added to medium at higher concentration (12).

5. CONCLUSION

M. luteus and *Staphylococcus* spp. used here produce extracellular penicillin acylse. Production of enzyme is better at elevated temperature. Enzyme of both tolerated higher temperature but pH should be near neutrality. Presence of glucose and sucrose at small concentration stimulated enzyme production by *M. luteus* and *Staphylococcus* spp respectively. Penicillin acylase from both these bacteria has broad substrate specificity even Penicillin G was added to production medium as inducer for enzyme production. And surprisingly the enzyme was more active on ampicillin rather than other beta lactum antibiotics tested.

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