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Research Paper

Lipase from organic solvent tolerant Bacillus strain C5: Isolation and Identification

Biochemistry

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B. Rajesh Dept. of Biochemistry, Institute of Science, GITAM University, VSKP

I. Bhaskar Reddy Dept. of Biochemistry, Institute of Science, GITAM University, VSKP

ABSTRACT

Organic solvents are extremely toxic to microbial cells even at very low concentration of 0.1% (v/v). They disrupt the bacterial cell membrane, affecting the functional and structural integrity of the cells The aim of the present work was to develop a bioprocess using specific and sensitive plate assay for true lipase producing bacterium capable of tolerating various organic solvents from spoiled coconut. The bacteria was identified as Bacillus species and it showed tolerance to both aromatic and aliphatic solvents with maximum tolerance for n-hexane. Bacteria showed maximum growth at 45°C, pH 8 and retained more than 80% of its initial activity upon exposure to various organic solvents.

1. Introduction:

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) constitute one of most important groups of industry enzymes, for they are being increasingly exploited for all kinds of fields including detergents, dairy, diagnostics, oil and lipid processing, and biotransformation [9]. Usually lipases catalyse the hydrolysis of water-insoluble triglyceride at an oil-water interface to release di-acylglyceride and mono-acylglyceride, free fatty acid and glycerol, while in immiscible or anhydrous solvents lipases accelerate other chemical reactions such as esterification, transesterification, aminolysis, acidolysis, and alcoholysis[1].

Lipases are present in microorganisms, plants and animals. Microbial lipases are becoming interest due to their potential as biotechnological catalysts, as well as by their role as virulence factors in some pathogenic organisms [2]. The bacterial lipases have been extensively studied mainly due to the industrial or clinical interest of these enzymes [3]. Use of enzyme in organic media has exhibited many advantages: increased activity and stability; regiospecificity and steroselectivity; higher solubility of substrate and ease of products recovery; ability to shift the reaction equilibrium toward synthetic direction [4]. Therefore, search for organic solvent-tolerant enzymes has been an extensive area of research [5].

Enzymes are generally not stable in the presence of organic solvents and are apt to denature and inactivated, most organisms lose their functions and cease growing. Some organic solvent-tolerant bacterial strains have been reported in recent years [6]. In a similar effort, we have isolated a bacterial strain which tolerates organic solvents to a certain degree and will be very useful in industry. The present paper describes isolation and identification of organic solvent-tolerant bacteria having lipolytic activity.

2. Materials and Methods:

2.1 Microorganism:

An alkaline thermo stable lipase from organic solvent tolerant bacterial strain was isolated from sun dried spoiled coconut and identified as Bacillus strain C5 was used for the present study. It was maintained by monthly subculturing at 37ºC and stored at 4ºC.

2.2 Lipase Plate Assays:

2.2.1 Rhodamine B - Olive oil Agar Medium:

The screening medium containing 0.8% of nutrient broth, 0.4% of sodium chloride, 2% of agar-agar medium, was adjusted to pH 7, autoclaved and cooled to about 60ºC. Then add sterilized 3% olive oil and 1ml Rhodamine B(10mg/ml) with vigorous stirring and emulsified by mixing for 1 min. and allowed to stand for few minutes to reduce foaming[7]. Aliquots of 20 ml were poured into each petridish to solidify. Each culture from daughter slants was streaked and incubated at 45ºC for 48 h. The lipase-producers were identified by the presence of orange colonies, whereas non lipase-producers were identified by the presence of pink colonies.

2.2.2 Tributyrin Agar Medium:

This screening medium was supplemented with 0.5%(w/v) peptone, 0.3%(w/v) yeast extract, 0.1% (v/v) tributyrin , 2% agar, pH 7.5[8]. After streaking, plates were incubated at 37ºC for 48 h. Then diameter of the colonies (d) and the diameter of the total clear hydrolytic halos including the colonies (D) were determined. The strains that yielded higher halos (D-d) were selected for furture studies.

2.3 Screening and isolation of organic solvent lipase producing bacteria:

Bacterial organisms were isolated from sun dried spoiled coconut using nutrient agar medium by dilution technique [9]. Each isolate was tested for its true lipase production on Rhodamine B-Olive oil agar medium. Positive colonies were selected for furture screening of potential organic solvent tolerant bacteria by streaking on tributyrin agar medium upon which each plate was flooded with 7ml of different organic solvents such as acetone, benzene, toluene, ethybenzene, 1-butanol, n-hexane and n-heptane and incubated at 45ºC for 48h [10]. The ability of the isolates to grow and produce lipase was observed and diameter of clearance zone of colony showing maximum hydrolysis was measured and selected for furture studies.

2.4 Morphological studies on isolated bacterium:

The selected strain was identified following Bergey's manual of Determinative Bacteriology [11].

2.5 Growth and lipase production profile:

The single colony of the pure culture C5 was inoculated into nutrient tmedium and cultivated on a rotatory shaker at 120 r/min and 37°C. The 250 ml Erlenmeyer flasks containing 100 ml tributyrin broth [1% (v/v) tributyrin oil, 0.5% (w/v) peptone and 0.3% (w/v) yeast extract] were inoculated with an overnight culture of C5 to obtain an initial culture density (OD 600 nm) 0.05 and incubated on rotator shaker at 200 r/min at 45°C. The samples were withdrawn at regular time interval of 4 h and analyzed for cell growth and enzyme activity.The enzyme activity was estimated from the supernatant obtained upon centrifugation of 10ml medium at 10000 r/min for 15 min.The cell pellet obtained upon centrifugation was resuspended in 10 ml of distilled water and its absorbance measured at 600nm and reported as growth of the culture.

2.6 Analytical Methods:

2.6.1 Lipase Assay:

To detect the activity of lipase produced by bacteria, the reaction mixture containing olive oil emulsion (composed of 25 ml olive oil and 75 ml 2% polyvinyl alcohol solution, 4 ml of 0.2 M tris buffer, 1 ml of 110mM CaCl2 and 1 ml enzyme solution) was used[12]. After incubation in orbital shaker at 200 r/min and 45ºC for 10 min, the enzyme activity was stopped by adding 20 ml of acetone ethanol (1:1) mixture and liberated free fatty acid was titrated against 0.02 M NaOH using phenolphthalein as indicator. Blanks were measured with a heat-inactivated enzyme sample, for which an enzyme stock solution was kept

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at 100°Cfor 15 min. After cooling to ambient temperature, the solution was used as described for the active enzyme sample. One unit of lipase was defined as the amount of enzyme, which liberates 1 mol of fatty acid/min[13].

2.6.2 Protein Assay:
Protein concentration was determined according to the method of Bradford protein micro assay [14]. Bovine serum albumin (BSA) solutions at concentrations from 0 to 50 µg/ml were used as standards.

3. Results and Discussions:

3.1 Isolation and selection of organic solvent lipase producing organism:

Among numerous isolated bacteria, ten strains showed better lipolytic activity. Lipase producer strains were identified by the formation of orange colonies when olive oil-rhodamine B spread plates incubated at 45°C for 48 hr. Organic Solvent tolerance of the isolates was investigated by their ability to grow on tributyrin agar plates flooded with different solvents (acetone, benzene, toluene, ethybenzene, 1-butanol, n-hexane and n-heptane) during incubation. The C5 strain exhibited growth as well as lipase production in presence of all the above solvents tested with maximum zone of hydrolysis on plates flooded with n-hexane (19 mm) shown in Table 1 and hence it was selected for future studies.

Table 1: The diameter ratio of clear zone and colony of the isolated Organic solvent tolerant bacteria.

Microorganisms	Diameter ratio of clear zone and colony (D-d) in presence of n-hexane	Lipase Activity (IU/ml)
C2	13mm	4.0
C5	19mm	4.6
C9	11mm	3.5
C15	13mm	3.9
C21	16mm	4.2
C28	12mm	3.7

3.2 Identification and taxonomical studies of isolate C5:

Taxonomical characteristics of the strain C5 are shown in Table 2. The colony appeared to be irregular, cloudy, smooth, moist, raised, entire and opaque on nutrient agar medium. The bacterium is a Gram-positive rod, formed spore and arranged in 2-3 chains. It was able to hydrolyse gelatine, casein, triolein and produce gas and acid in glucose and sucrose broth. The above showed a typical characteristic of the genus Bacillus.

Table 2: Taxonomical characteristics of strain C5

Parameters	Characteristics
Colony Morphology Size Colour Form Surface Texture Elevation Margin	 Medium Cloudy Irregular Shine & Smooth Moist Raised Entire
Microscopic Observation Gram stain Shape Size Arrangement Motility KOH test Spore position	 Gram Positive Rods Medium 2-3 chains Negative Negative Terminal
Oxygen requirement	Facultative
Growth at Different temperatures Room temperature 37 °C 45 °C 65°C	 Positive Positive Positive Negative
Growth at Different pH pH <5 pH >5.8 to 9 pH >9	 Negative Positive Negative
Growth at Different NaCl(%) 2.5% 5% 7%	 Positive Positive Negative
Biochemical Characteristics Oxidase test Catalase test Nitrate test Hydrogen sulfide formation Methyl red test Vogue–Proskauer test Indole production Urease activity Citrate utilization	 Positive Positive Negative Positive Negative Negative Negative Positive Negative
Hydrolysis of Tributyrin Triolein Gelatin Tween 80 Starch Casein	 Positive Positive Positive Positive Positive Negative
Carbon sources for growth Starch Raffinose Sucrose Lactose Maltose Trehalose Cellobiose Melibiose Ribose Arabinose Xylose Rhamnose Dextrose Manose Galactose Fructose Adonitol(ribitol) Mannitol Sorbitol Dulcitol(galacitol) Inositol Myo-Inositol Salicin	 Negative Negative Positive Negative Negative Positive Negative Negative Negative Negative Negative Negative Positive Positive Negative Positive Negative Negative Negative Negative Negative Negative Positive
Special Tests Esculin ONPG	 Positive Positive

Note: Tests were repeated three times

3.3 Growth and lipase production profile:

The ability of Bacillus strain C5 for production of lipolytic enzyme was measured in liquid medium.Maximum lipase activity of 4.6 IU/ml was observed at 44th hr of production with OD 1.53 at 600 nm. On third day, increase in lipase activity is negligible and after third day, decline in lipase activity was observed. It was observed that C5 exhibited lipase production at 45°C, 200 rpm and an initial pH 8.0.Therefore,these conditions were employed for cultures used in future experimentation.

4. Conclusion: In the present study an alkaline lipase producing bacteria was isolated from spoiled coconut and identified by morphological and biochemical characterization. The bacteria was identified as Bacillus species and it showed tolerance to both aromatic and aliphatic solvents with maximum tolerance for n-hexane.

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