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Isolation and Optimization of Proteolytic and Lipolytic Bacteria from Dairy Effluent

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The present study aimed at optimization of the four best isolates selected from both proteolytic (out of 30) and lipolytic (out of 21) based on the maximum zone of clearance. The potential bacterial strains were sequenced and identified as L2 - *Lysinibacillus sphaericus*, L8 - *Pseudomonas taiwanensis,* P11 - *Bacillus marisflavi* and P9 - *Pseudomonas aeruginosa* by 16s rRNA analysis. Optimization of Protease Enzyme Activity (P9, P11) and Lipase Enzyme Activity (L2, L8) were studied. Peak proteolytic activity for P9 was observed for carbon source as maltose, yeast extract as nitrogen source, wheat bran as substrate, at pH 7.0 and the isolate P11 revealed optimum carbon source as fructose, beef extract as nitrogen source, whey as substrate, at pH 8.0. Similarly, peak lipolytic activity for L2 was observed for carbon source as sucrose, urea as nitrogen source, at pH 7.0, palm oil as substrate and the isolate L8 resulted maltose as carbon source, tryptone as nitrogen source, at pH 7, palm oil as substrate. From the results of the present study, it could be inferred that the isolates from the dairy effluent can be used for effective biological method of the treatment thereby supporting the degradation of organic and inorganic nutrients.

Key words: Bacterial isolates, Dairy effluent, Proteolytic bacteria, Lipolytic bacteria, Enzyme units.

Environmental protection and waste minimization using biotechnological processes is an emerging discipline and holds key theory of the modern industrial society. The treatment of industrial effluent depends on its quality, quantity and the source of receiving medium and the dilution available.

The dairy industry is one of the most important food industries which generate high strength effluent with high BOD and COD concentration. They are primarily generated from the cleaning and washing operations in the milk processing plants[1]. The volume of effluent from a dairy industry generates about 0.2 – 10 liters of effluent per liter of processed milk.

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Some physicochemical processes used for the treatment of dairy effluent such as Osmosis, Ultrafiltration, electrochemical treatment are invariably cost intensive from either the use of external acid sources or flocculating agents. Compared to chemical/physical methods, biological processes are more advantageous because of their cost effectiveness, eco friendliness and lower sludge production. The dairy effluent is highly bio-degradable and can be treated with biological unit processes effectively.

The effluent of dairy contain large quantities of milk constituents such as lipids, lactose, casein, inorganic salt, besides sanitizers and detergents used for washing[2]. Lipases are a group of enzymes that catalyze the hydrolysis of triacylglycerols to diacylglycerol, monoacyl-glycerol, fattyacids and glycerols at the interface between aqueous and the lipid phase. Proteases or peptidyl-peptide hydrolases are enzymes that carry out proteolysis by hydrolysis of the peptide

bonds that link amino acids together in the polypeptide chain.

Hence the present study was aimed to isolate protease and lipase bacteria from dairy effluent and optimize the best strains which could be employed for proficient biological treatment of dairy effluent.

MATERIALS AND METHODS

Sample Collection

The dairy plant sludge sample and effluent was collected from the Aavin Dairy plant, Chennai in a sterilized container and brought to the laboratory in icebox for immediate processing. Half strength nutrient agar medium with 0.5% Tween 80 (substrate for lipase) and 0.5% Casein (substrate for protease) respectively were used for plating the serial diluted sample. The isolate demonstrating the zone of clearance (proteolysis) and opaque zone (Lipolysis) from the corresponding plates were selected for further study.

Screening of the isolates (qualitative plate assay)

The purified cultures were screened by streaking them on skim milk agar plates for proteolytic activity and tributyrin agar base plates for lipolytic activity. The zone of hydrolysis was measured to the nearest mm. The isolates with maximum zone of hydrolysis were maintained on minimal agar slants (Yeast Extract, NaCl, peptone and 2% Agar, pH 7.0) at 4 C and were subcultured every 15 days.

Identification of the isolates

The bacterial strains were identified by 16s r RNA analysis from PCBS (Pondicherry Center for Biological Sciences), Pondicherry.

Optimization of culture conditions

The isolates were grown in the mineral salt medium supplemented with different carbon and nitrogen sources. Effects of various physical parameters such as temperature, initial pH and incubation period were also optimized by conventional methods for maximum enzyme activity. All the experiments were conducted three times.

Determination of Protease Activity

The protease activity was estimated using Universal Protease assay [3]. 1ml of CFS (Cell Free Supernatant) was added to 1ml of 1% (W/V) casein solution in glycine – NaOH buffer of pH 10.5 and incubated for 10min at 60 °C. The reaction was stopped by addition of 4ml of 5% trichloro acetic acid. The reaction mixture was then centrifuged at 3000 rpm for 10 min and to 1 ml of the supernatant; 5ml of 0.4M Na2Co3 was added followed by 0.5 ml Folins – Ciocalteu reagent. The amount of tyrosine released was determined using UV Spectrophotometer at 660nm against the enzyme blank. One unit of protease activity was defined as "the amount of enzyme required to release 1µg of tyrosine per ml per min under standard assay conditions".

Calculation

$$\text{Protease activity (units/ml)} = \frac{\mu \text{ mole tyrosine equivalents released} \times \text{Total volume of assay}}{\text{Time of assay} \times \text{Volume of enyzme} \times \text{Volume used in spectrophotometric determination}}$$

Determination of Lipase Activity

The lipase activity was estimated by titrimetric method using olive oil as a substrate [4]. One ml sample solution was added to the assay substrate containing 10ml of 10% homogenized olive oil I 10% gum acacia, 2ml of 0.6% $CaCl_2$ solution and 5ml of 0.2 mol/lit phosphate buffer pH 7.0. The enzyme mixture was incubated on an orbital shaker with a shaking speed of 100 pm at 37 °C for 1hr. To stop the reaction, 20ml of ethanol : acetone mixture (1:1) was added to the reaction mixture. The fatty acids liberated were titrated with 0.1 N NaOH using phenolphthalein as an indicator. The end point was light pink in color. One unit of lipase is defined as "the amount of enzyme which releases one micromole fatty acid per minute under specified assay conditions".

Calculation

$$\text{Lipase activity (units/ml)} = \frac{\Delta V \times N \times 1000}{V \text{ (Sample)} \times 60}$$

$\Delta V = V2 - V1$

V1 = Volume of NaOH used against control flask

V2 = Volume of NaOH used against experimental flask

N = Normality of NaOH

V (sample) = Volume of enzyme extract

RESULTS

Isolation and screening of proteolytic and lipolytic bacterias

A total of 30 proteolytic and 21 lipolytic bacterias were isolated from the dairy sludge

examined. When tested for their proteolytic potential, 8 isolates (P9, P11, P13, P15, P16, P22, P23, and P24) demonstrated clear zones on the skimmed milk agar, out of which P9 and P11 were selected for further study analysis. Similarly, lipolytic isolates (L2 and L8) demonstrated clear zones on the tributyrin agar and selected for further analysis (Figure 1a, 1b).

Identification of the selected bacterial isolate

The morphological characters and biochemical characterization has been studied and presented in table 1 and 2.The isolates were sequenced and identified as P9 – Pseudomonas aeruginosa, P11 – Bacillus marisflavi, L2 – Lysinibacillus sphaericus, L8 – Pseudomonas taiwanensis by 16s rRNA analysis.

Optimization of culture conditions

Effect of carbon source

Among the various carbon sources, the best carbon source for proteolytic bacteria: P9 - Maltose (290.34 U/ml) and P11 - Fructose (39.01 U/ml) were observed. Similarly, the best carbon source for lipolytic bacteria: L2 - Sucrose (72.18 U/ml) and L8 – Maltose (35.30 U/ml) were reported (Figure 2a, 2b).

Effect of nitrogen source

Various nitrogen sources were investigated for protease and lipase production. The maximum amount of enzyme production was obtained in Yeast Extract (327.89 U/ml) - P9 and Beef Extract (38.01 U/ml) - P11 for proteolytic bacteria. Similarly in lipolytic bacteria, Urea (76.2 U/ml) - L2 and Tryptone (29.2 U/ml) – L8 showed best results (Figures 3a, 3b).

Table 1. Morphological characteristic of the potential bacterial isolates

Characters	P9	P11	L2	L8
Size	3.5mm	2mm	1.5mm	1.1mm
Shape	circular	circular	long rods	circular
Margin	Regular	Filamentous	Regular	Entire
Opacity	Translucent	Opaque	Opaque	Translucent
Consistency	Moist	Moist	Moist	Moist
Gram strain	Gram negative	Gram positive	Gram positive	Gram positive
Motility	Motile	Motile	Motile	Motile

Table 2. Biochemical tests of the potential bacterial isolates

Terms	P9	P11	L2	L8
Catalase	+	+	+	+
Oxidase	+	-	+	+
Indole	+	-	-	+
MR	+	-	-	+
VP	-	-	-	-
Citrate	+	-	-	-
Urease	+	-	-	+
H2S	-	-	+	-
Nitrate Reduction	+	+	-	+
HL	+	-	-	-
Lactose	-	-	-	-
Glucose	+	+	-	-
Sucrose	+	+	-	-
Maltose	-	+	-	-
Mannitol	-	+	-	-
Inositol	+	+	+	-
Xylose	-	+	-	-
Gelatinase	+	-	+	+
Amylase	+	-	-	+
caseinase	+	+	-	-

Effect of substrate

Maximum lipase production was observed in wheat bran (283.25 U/ml) – P9; whey (36.22U/ml) - P11 and maximum protease production was observed in palm oil (75.3 U/ml) – L2; (35.7 U/ml) – L8 after 48h of incubation at 37 °C (Figures 4a, 4b).

Effect of pH of the medium

The effect of various pH on protease and lipase production was recorded. The maximum activity was observed for proteolytic bacteria at pH7 (P9) and pH8 (P11). Similarly, at pH 7 maximum lipase activity of L2 (63.7 U/ml) and L8 (25.11 U/ml) was observed.

Effect of temperature

The optimum temperature for protease (P9 – 360.17 U/ml, P11 – 39.42 U/ml) and lipase

production of (L2 – 47.05 U/ml, L8 – 36.12 U/ml) was found to be 37 ° C.

Effect of incubation time

The maximum amount of protease production was observed in 48hrs incubation time (351.21 U/ml) for P9 and (32.13 U/ml) for P11 and similarly maximum lipase production was observed in (49.44 U/ml) for L2 and (35.2 U/ml) for L8.

DISCUSSIONS

Lipases are ubiquitous enzymes that catalyze the hydrolysis of fats and oils. Due to this reason, they have high biotechnological potential and currently attracting enormous attention [5]. In all living organisms, proteolytic enzymes are widely found and are essential for cell growth and differentiation [6]. High yield of enzyme produced from carbon sources such as maltose [8] and sucrose have been reported by other researchers. Afify et al., [7] concluded that the isolate could produce protease which is extracellular and utilized maltose as solitary carbon source for protease production. Our observation is in agreement with it.

In parallel to our findings, an earlier study concluded that organic nitrogen sources such as peptone, yeast extract and beef extract had significant effects on extracellular protease production by a halophilic Bacillus sp. [11]. On the other hand, beef extract among the different nitrogen sources led to a proteolytic activity by Bacillus sp.[12].According to Sundaramoorthi et al.,[13] the maximum lipase activity was achieved at 48hrs of incubation period by using palm oil as substrate which completely agrees with the present finding. Likewise, the maximum protease activity was observed using wheat bran as the substrate on Bacillus subtilis isolated from dairy effluent [14] which correlates our findings.

Srinivasan et al., [15] characterized the thermostable protease producing bacteria from tannery industry effluent and reported that the optimum yield was achieved after 48hrs with the pH 8.0 which matches with our observation. It is

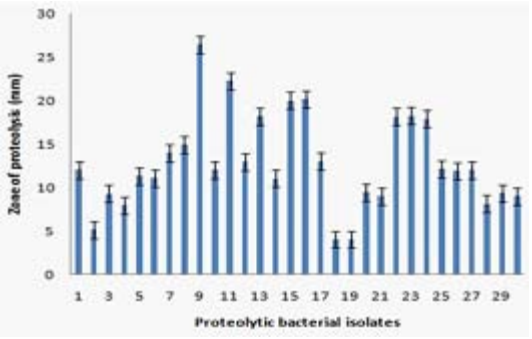

Fig. 1a. Zone diameter of proteolytic activity by the bacterial isolates. Data represent mean ± S.D. (n=3); $P < 0.05$

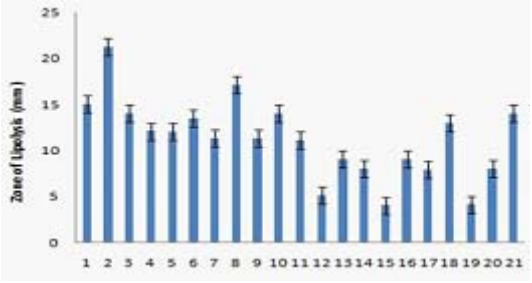

Fig. 1b. Zone diameter of lipolytic activity by the bacterial isolates. Data represent mean ± S.D. (n=3); $P < 0.05$

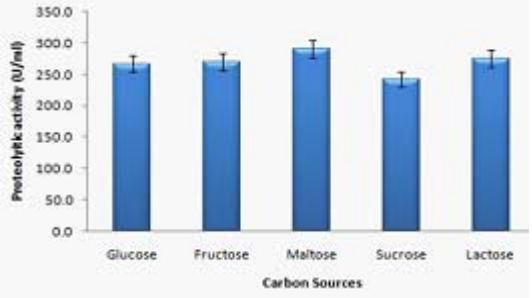

Fig. 2a. Effect of carbon source on protease production. Data represent mean ± S.D. (n=3); $P < 0.05$

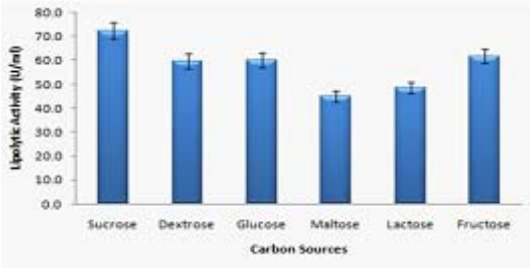

Fig. 2b. Effect of carbon source on lipase production. Data represent mean ± S.D. (n=3); $P < 0.05$

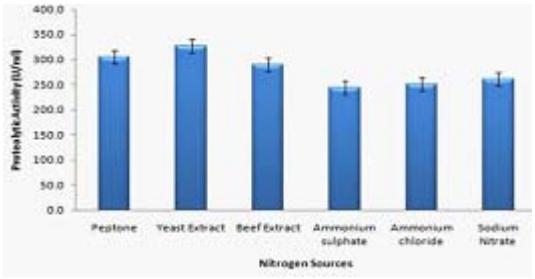

Fig. 3a. Effect of Nitrogen source on protease production. Data represent mean ± S.D. (n=3); $P < 0.05$

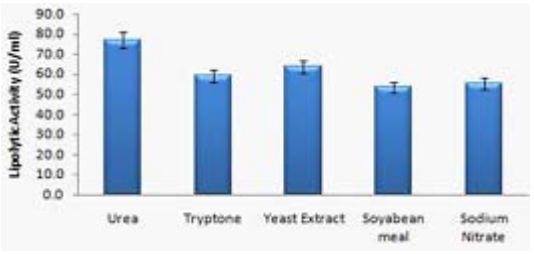

Fig. 3b. Effect of Nitrogen source on lipase production. Data represent mean ± S.D. (n=3); $P < 0.05$

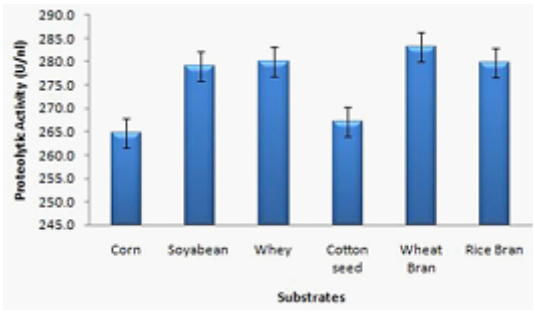

Fig. 4a. Effect of Substrates on protease production. Data represent mean ± S.D. (n=3); $P < 0.05$

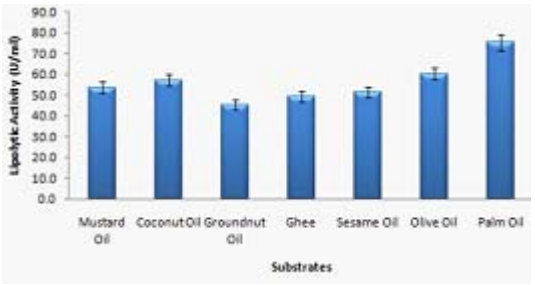

Fig. 4b. Effect of Substrates on lipase production. Data represent mean ± S.D. (n=3); $P < 0.05$

very essential to detect the optimum incubation period for which an organism shows maximum enzyme activity because organisms show differences at various incubation periods [16]. Our study ties with the reports of previous researchers who observed high lipolytic activity at 48hrs at the stationary phase and gradually decreased after 48 hrs [9, 10].

CONCLUSION

In this study, the proteolytic and lipolytic bacterias were isolated and identifed as L2 - *Lysinibacillus sphaericus*, L8 - *Pseudomonas taiwanensis*, P11 - *Bacillus marisflavi* and P9 - *Pseudomonas aeruginosa* and the optimum values of the parameters such as pH, temperature, incubation time, substrate concentration,carbon and nitrogen sources for the maximum enzymatic activities were investigated. All the above test results indicate the potential use of the microorganism as biotechnological tools and further studies can be focused on using microbial mixture of these isolates for dairy effluent treatment.

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