Electronic Journal of Biotechnology 18 (2015) 20–28

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Electronic Journal of Biotechnology

Utilization of coconut oil mill waste as a substrate for optimized lipase production, oil biodegradation and enzyme purification studies in *Staphylococcus pasteuri*

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ARTICLE INFO

Article history:
Received 30 July 2014
Accepted 5 November 2014
Available online 28 November 2014

Keywords:
Bioremediation
Fat and oil contaminants
Ion exchange chromatography
Lipase characterization
Response surface methodology

ABSTRACT

Background: Oil and grease laden wastewaters pose hindrance to the treatment units and further threaten the receiving water bodies. Lipase-producing microbial strains are increasingly being exploited for the remediation of such effluents.
Results: When bacterial strains isolated from oil mill effluent were screened for their lipolytic activity, two isolates, COM-4A and COM-6B showed significant extracellular lipase activity. They were identified to be *Staphylococcus pasteuri* and *Bacillus subtilis*, respectively. *S. pasteuri* COM-4A was cultivated in nutrient media based on coconut oil mill waste (CMW), in which it showed good growth at concentrations up to 20 g/L. While growing in such media, it was capable of producing lipase and other important extracellular hydrolytic enzymes. Furthermore, the isolate was able to effectively biodegrade the CMW supplemented in the medium. Applying the Box Behnken Design of Response Surface Methodology, lead to a 1.4-fold increase in both lipase production and oil removal by the isolate. The lipase was purified 9.02-fold and the molecular weight of the monomeric enzyme was deduced to be around 56 kDa. Characterization of the enzyme revealed it to be alkaliphilic and moderately thermophilic in nature, with pH and temperature optima of 9.0 and 50°C, respectively. The enzyme was also quite stable in the presence of water-miscible organic solvents.
Conclusion: Hence, the COM-4A lipase could be considered to be suitable for a variety of industrial applications such as in detergent formulations and in biodiesel production as well, apart from the possibility of applying it for bioremediation of fat and oil contaminants.

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1. Introduction

Oil and grease laden wastewaters are largely generated from food processing industries, oil mills, dairies, slaughter houses, restaurants, and bakeries. They consist predominantly of fatty acid esters of trihydroxy alcohol or glycerol. When released into receiving water bodies without adequate treatment, they pose serious threats to the sustenance of aquatic life therein. High oil and grease contents also cause severe obstacles in wastewater treatment plants where they result in interference of cell-aqueous phase transfer rates, sedimentation hindrance due to the growth of filamentous microorganisms, development of bulking sludge, clogging and emanation of foul odors [1]. Pretreatment of these wastewaters to bring about lipid hydrolysis makes them more amenable to conventional biological treatment and lipolytic microbial strains and the enzymes they secrete find promising applications in this sector [2]. Likewise, lipase catalyzed biodiesel production is an emerging area of green synthesis that offers advantages over conventional chemical synthesis [3]. Lipase mediated biopolymer synthesis is another noteworthy application of this versatile enzyme in the environmental domain [4].

Lipases (triacylglycerol acyl hydrolases, EC. 3.1.1.3) belong to the family of serine hydrolases. They catalyze the hydrolysis of esters formed from glycerol and long-chain fatty acids and the products released include di- and mono-acyl glycerols, fatty acids, and glycerol. Such ester hydrolysis is catalyzed in the aqueous environment and the reverse reactions of esterification, interesterification and transesterification are catalyzed in non-aqueous and micro-aqueous environments. Lipases are chemo-, regio- and enantio-selective in nature. Apart from their environmental applications, they also find widespread industrial relevance in food, detergent, textile, leather, paper and pharmaceutical sectors [5].

In our study, lipase was produced from *Staphylococcus pasteuri* isolated from oil and grease contaminated sites, using a medium based on coconut oil mill waste (CMW). This offers the environmental edge of exploiting a waste material as carbon source for culturing the microbe and producing the enzyme. Other important extracellular enzymatic activities of the microbe including protease and amylase activities were also investigated in this medium and statistical optimization of lipase production and oil biodegradation were performed. Lipase was then

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2014.11.003
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purified and characterized, shedding light on its properties and other applications for which it could prove beneficial.

2. Materials and methods

2.1. Chemicals

p-Nitrophenyl Palmitate, Phenyl Sepharose CL-4B, Phosphocellulose PC-11, molecular weight markers, bovine serum albumin, and Coomassie Brilliant Blue R-250 were procured from Sigma Chemicals, USA. All other reagents and chemicals used were of analytical grade.

2.2. Sample collection

Wasted oil residue after the extraction process (referred to as coconut oil mill waste) was collected from a coconut oil mill located in the Coimbatore District of Tamil Nadu, India and used to supplement the culture medium.

2.3. Screening and isolation of lipase producers

Bacterial strains were isolated from the oil mill effluent, which was serially diluted and the higher dilutions (10^{-5}–10^{-8}) were plated on nutrient agar supplemented with 2% (v/v) coconut oil and incubated overnight at 35°C. Discrete colonies were sub-cultured in the same medium and pure cultures were obtained. They were streaked on tributyrin agar (TBA) plates to screen for lipase activity. Lipase producers show a zone of clearance around the area of growth that is attributable to the butyric acid released upon tributyrin hydrolysis. Submerged fermentation in nutrient broth supplemented with 1% (v/v) coconut oil was further carried out in order to quantify the enzyme activity [6]. Protein content was also estimated [7].

2.4. Molecular identification

The two best isolates were identified using 16S rRNA gene sequence based molecular technique. Single colonies from freshly streaked agar plates were inoculated into nutrient broth. DNA was isolated and checked on agarose gel for purity and quantity. 16S rRNA gene primers 27F (5′-AGAGTTTGATC(A/C)TGGCTCAG-3′) and 1492R (5′ ACGG(C/T)TACCTTGTTACGACTT-3′) were used to amplify ~1.4 kb fragments from the isolated genomic DNA [8]. The amplified PCR (Polymerase Chain Reaction) products were gel purified, quantified and sequenced using an ABI prism 3100 Genetic Analyzer (Applied Biosystem). Sequence data obtained were analyzed and consensus sequences were generated from forward and reverse sequences using 'Aligner' software. These sequences were subjected to BLAST (Basic Local Alignment Search Tool) with NCBI (National Center for Biotechnology Information) GenBank database [9] and the cultures were identified. Phylogenetic trees were constructed using the neighbor joining method. Further studies were restricted to the single best lipase producer.

2.5. Cultivation of the bacterial isolate in CMW supplemented media

2.5.1. Estimation of biomass

Initially, experiments were carried out to ascertain the maximum concentration of CMW that could permit bacterial growth, as at very high concentrations, the oil layer interferes with oxygen transfer and impedes cell survival and growth. For this, 250 mL Erlenmeyer flask containing 100 mL of basal salt medium comprised of (g/L) $(NH_4)_2SO_4$ — 2.5, $CaCl_2$ — 1.0, $NaNO_3$ — 0.5, K_2HPO_4 — 0.5, and $MnSO_4$ — 0.05, was supplemented with varying concentrations of CMW ranging from 5.0 to 30 g/L (medium referred to as CMW-M1). The medium pH was adjusted to 7.0 ± 0.1 using 1 N NaOH or HCl, autoclaved, and inoculated (2% v/v) with overnight grown culture of the best lipase producing bacterial isolate (cell density of 42×10^4 CFU/mL). The culture was incubated at 35°C for 48 h, at an agitation rate of 120 rpm (Orbitek, India). Growth was measured by reading the absorbance at 600 nm in a UV–Visible Spectrophotometer (Cistronics, India). The culture was then centrifuged (Kubota 3700, Japan) at 10,000 g for 10 min and the pellet thus obtained was used to estimate biomass content after repeated washing and drying in hot air oven (NSW 151, India) overnight. Based on optical density readings and the amount of biomass obtained, the maximal concentration of CMW that permitted appreciable bacterial growth was ascertained.

2.5.2. Oil biodegradation

The residual oil content in the medium after bacterial growth and metabolism was also measured, in order to understand the degradative capability of the isolate. The percent reduction in the initial oil content (5.0–30 g/L) over a period of time (12–96 h) was estimated using the partition gravimetric method [10]. The culture supernatant was acidified to pH 2.0 with 1:1 diluted HCl and the residual oil content was repeatedly extracted with 30 mL of hexane until the aqueous phase showed no oil layer and the solvent phase appeared clear. The combined solvent extracts were evaporated completely at 70°C in a water bath (NSW 133, India) and the weight of the residual oil was measured, from which percent reduction was calculated.

Apart from the initial oil content and incubation time, the effects of other parameters including pH and temperature on the biodegradation rate were also studied by varying the medium pH from 3 to 12 and the incubation temperature from 30 to 70°C.

2.5.3. Extracellular enzymatic activities

For the isolate to be effectively used for bioremediation of oil industry effluent, it should be able to produce not only lipase for oil biodegradation, but also other important hydrolytic enzymes, so as to bring about an overall reduction in the effluent organic load. Enriching the oil waste supplemented minimal medium with other carbon and nitrogen sources may be helpful to spur microbial growth and activity. Hence, CMW-M1 was enriched with the following nutrients either alone or in different combinations (g/L): yeast extract — 1.5, peptone — 1.0, glucose/starch — 2.0. The media were inoculated and incubated as mentioned earlier, later the cultures were centrifuged and the supernatants were used to assay the extracellular enzymatic activities of lipase, protease and amylase. The standard protocols of Winkler and Stuckmann [6], Folin and Ciocalteu [11] and Bernfeld [12] were followed for performing these assays.

2.6. Statistical optimization of lipase production and oil biodegradation

Box–Behnken Design of Response surface methodology (RSM) was used to optimize lipase production and oil biodegradation by the best isolate. This optimization study was carried out using the Design Expert Software 8.0 (Stat Ease Inc. Minneapolis, U.S.A., trial version). CMW (A), peptone (B) and yeast extract (C) were the independent variables chosen for this purpose, the choice being made on the basis of results obtained in the preceding studies. These variables were studied at three different levels (−1, 0, +1) and a matrix encompassing 17 experiments was generated by the software. Production was carried out as per the design, under previously mentioned conditions. After the incubation period, lipase assay and residual oil extraction were performed using the cell-free supernatant. Enzyme activity and oil removal were recorded as responses R1 and R2, respectively. These data were fed into the software and analyzed. Standard analysis of variance (ANOVA) and contour plots were generated. A quadratic polynomial regression model was assumed for

the predicted response. For a three-factor system, the model equation is as follows:

$$Y = \beta_0 + \beta_1 A + \beta_2 * B + \beta_3 * C + \beta_{11} * A^2 + \beta_{22} * B^2 + \beta_{33} * C^2 + \beta_{12} * AB + \beta_{13} * AC + \beta_{23} * BC$$

[Equation 1]

where, Y is the predicted response, β_0 is the intercept, β_1, β_2, and β_3, are the linear coefficients, β_{11}, β_{22}, and β_{33}, are the squared coefficients, and β_{12}, β_{13}, and β_{23}, are the interaction coefficients.

2.7. Lipase purification

CMW supplemented medium was inoculated with 2% (v/v) seed culture of the bacterial isolate (overnight grown, cell density 56×10^4 CFU/mL) and cultured for 48 h at 35°C. It was then centrifuged and the clear supernatant was subjected to 75% $(NH_4)_2SO_4$ cut and left overnight at 4°C. It was further centrifuged and the pellet was suspended and dialyzed twice against equilibration buffer containing 10 mM KPO_4 pH 7.4, 1 mM EDTA, 1 M $(NH_4)_2SO_4$ and 7 mM 2-mercaptoethanol.

The protein was purified using Phenyl Sepharose CL-4B matrix. The column was equilibrated using ten bed volumes of equilibration buffer. The sample was loaded, the column was washed with five bed volumes of the buffer and subsequently step elution was performed using a buffer of similar composition, but containing decreasing salt concentrations (0.6 M, 0.3 M and 0 M). 1 mL fractions were collected, they were assayed for lipase activity and the active fractions were pooled. The pooled fractions were applied onto ion exchange column packed with Phosphocellulose PC-11. This column was equilibrated using ten bed volumes of equilibration buffer containing 10 mM KPO_4 pH 7.4, 1 mM EDTA, 50 mM KCl and 7 mM 2-mercaptoethanol. The sample was loaded, and after washing the column with five bed volumes of the same buffer, it was eluted using a gradient of low to high salt buffer (equilibration buffer containing 0–1.0 M KCl). 16 fractions of 1 mL each were collected, assayed for lipase activity, the active fractions were pooled, dialyzed and lyophilized.

The samples obtained from each stage of the purification process were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% separating gel [13], to check for purity and to estimate molecular weight. The protein bands were stained using Coomassie Brilliant Blue R-250.

2.8. Characterization studies

2.8.1. Effect of pH

Effect of pH on lipase activity was studied using substrate solutions prepared with buffers of pH values ranging from 3 to 12. For the stability study, enzyme solution was pre-incubated with equal volumes of pH 3–12 buffers for 1 h, and the residual activity was assayed under optimal conditions.

2.8.2. Effect of temperature

Effect of temperature on lipase activity was determined by incubating the assay mixture at various temperatures ranging from 20 to 80°C and the activity units were recorded. For the stability study, the enzyme solution was pre-incubated at various temperatures ranging from 20 to 80°C for 1 h, and the residual activity was measured under optimal conditions.

2.8.3. Effect of metal ions, detergents, and organic solvents

To assess lipase stability, purified enzyme solution was pre-incubated with an equal volume of 100 mM KCl, $MgSO_4$, $CuSO_4$, $CaCl_2$, $FeCl_3$ and $AgNO_3$ for 1 h, and the residual activity was determined under optimal conditions. The enzyme sample was then pre-incubated with an equal volume of various detergents such as 1% (v/v) SDS, tween-20, triton X-100, sodium taurocholate, deoxycholate, and CTAB for 1 h, and the residual activity was assessed. Next, it was pre-incubated for the same duration with 10% organic solvents of ethanol, methanol, isopropanol, n-hexane, toluene, chloroform and residual activity was determined.

Fig. 1. Phylogenetic tree of *S. pasteuri* strain COM4A.

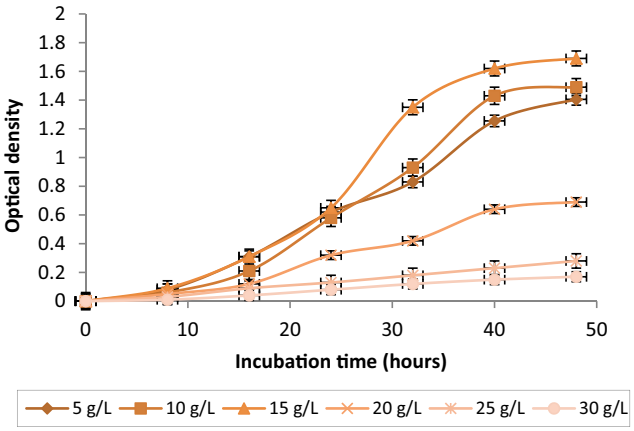

Fig. 2. Bacterial growth in basal salts medium supplemented with varying concentrations of CMW.

3. Results and discussion

3.1. Screening and isolation of lipase producers

When the effluent samples were serially diluted and plated in a nutrient medium that enriched lipolytic organisms, 23 bacterial strains were isolated. They were subsequently inoculated in tributyrin agar plates and zone of clearance was seen around 12 colonies. When p-NPP assay was carried out to quantify their lipase activity, seven isolates showed promising results. Among them, the strain COM-4A exhibited the highest enzyme activity of 18.23 U/mL and specific activity of 1.34. This was followed by the strain COM-6B, which showed 15.89 U/mL of lipase activity and a specific activity of 0.93.

3.2. Molecular identification

Sequencing of the 16S ribosomal RNA gene and analyzing the generated sequences using BLAST revealed that COM-4A belonged to the genus *Staphylococcus*. 100% homology to previously deposited sequences of *S. pasteuri* strains was observed. Although other species of *Staphylococci* such as *Staphylococcus aureus* [14], *Staphylococcus xylosus* [15], *Staphylococcus simulans* [16] and *Staphylococcus warneri* [17] have been reported for lipase activity, *S. pasteuri* could be considered to be a novel source of bacterial lipase, thus making our finding significant and worthy of further investigations. The other isolate COM-6B was identified to be *Bacillus subtilis*. The phylogenetic tree of COM-4A is presented in Fig. 1. These sequences have also been submitted to NCBI GenBank and have been allotted Accession Numbers KJ020928 (*S. pasteuri*) and KJ886969 (*B. subtilis*).

3.3. Cultivation of the bacterial isolate in CMW supplemented media

3.3.1. Estimation of biomass

Supplementing the basal salt medium with CMW spurred bacterial growth up to a particular point, beyond which it was inhibitory to the cells. Increasing the CMW concentration from 5.0 to 15 g/L, led to an increase in the optical density recorded. However, when the CMW supplementation in the medium went up to 20 g/L, the culture turbidity slightly dipped and further increase in the CMW concentration (25 g/L) severely retarded bacterial growth as reflected by a very low OD value of 0.17, after 48 h of incubation (Fig. 2). The biomass dry weight measured after a similar incubation period was also quite low (0.16 g/L) at such a high concentration. From these results, it was concluded that 15 g/L is an effective concentration of CMW for supplementing the growth medium, although up to 20 g/L was reasonably well tolerated by the organism.

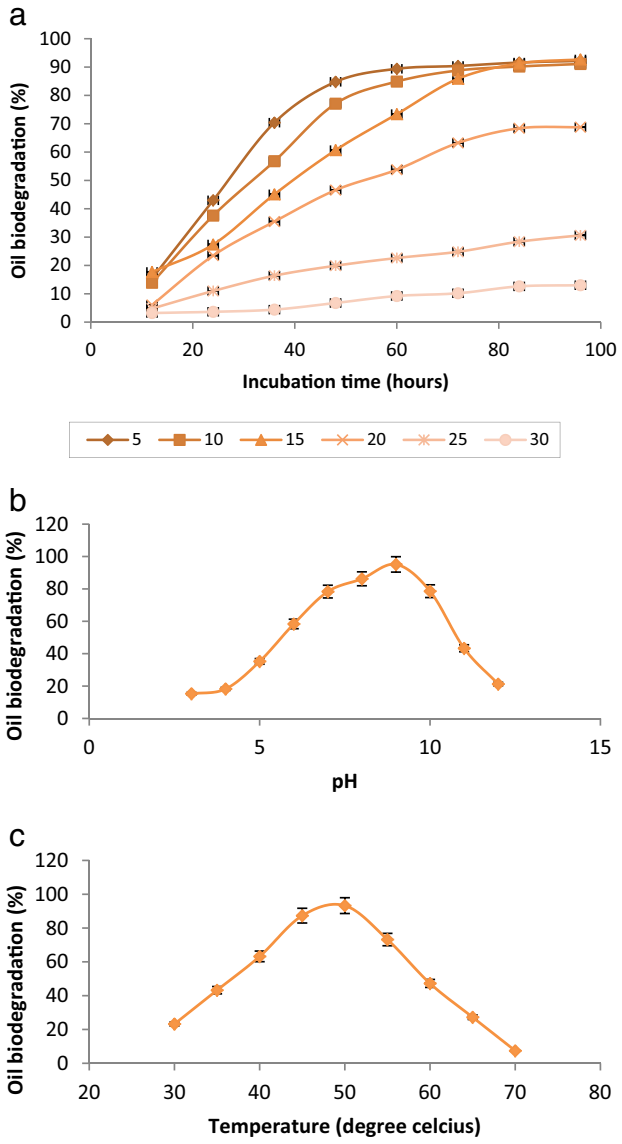

Fig. 3. Effects of different parameters on CMW biodegradation. (a) Effects of initial oil concentration (g/L) and incubation time, (b) effect of pH, and (c) effect of temperature. The studies in panels b and c were performed using an initial CMW concentration of 15 g/L, the maximum concentration that had yielded good growth and biodegradation. The incubation time was fixed at 72 h, since there was only negligible increase in biodegradation beyond that.

3.3.2. Oil biodegradation

Investigations on oil biodegradation by the isolate also yielded comparable results. The removal efficiency was found to be over 90%, when the initial concentration of CMW in the medium was up to 15 g/L. This fell down to 68% at a concentration of 20 g/L and plunged down to 13% at a concentration of 30 g/L (Fig. 3a). This low biodegradation rate is expected at such high concentrations of CMW, as the growth itself was scanty and the degradative activity would also be accordingly low. In a comparative study involving biodegradation of lipid-rich wastewater using several lipase-producing bacterial species, *S. aureus* had shown significant lipid degradation in slaughter house wastewater [18].

Studies on the effect of pH revealed maximum biodegradation to occur at an alkaline pH of 9, with good results being observed in the range of 7–10. When the effect of temperature on the process was studied, an increase in temperature was noted to favor biodegradation, up to a maximum of 50°C, beyond which, further increase in

temperature impaired the process (Fig. 3b). Hence, moderate thermo-alkaliphilic conditions could be inferred to be conducive for oil biodegradation, which could be the result of better lipase activity under these circumstances.

3.3.3. Extracellular enzymatic activities

When the extracellular enzymatic activities of the isolates in different culture media were compared (Fig. 4), they were observed to be relatively low in CMW containing basal salts medium (CMW-M1). However, even this minimal medium resulted in appreciable lipase activity of 9.95 units, although other extracellular enzymes were seen to be produced at negligible levels. Augmenting this medium with yeast extract and peptone (CMW-M5) accelerated the lipase production to 22.28 units. Presence of glucose in the medium seemed to have an inhibitory effect on lipase production. Protease activity was also recorded to be the highest at 22.9 U/mL in CMW-M5 and quite close in the medium that additionally contained glucose (CMW-M8). Amylase activity was generally noted to be high in all starch-supplemented media, with the highest activity of 24 U/mL recorded in CMW-M8, thereby indicating its inducible nature. Combined hydrolytic activities in *S. xylosus* strain MHB32, which was able to produce an array of hydrolytic enzymes including protease — 77 U/mL, amylase — 56 U/mL, L-asparginase — 43 U/mL, lipase — 25 U/mL, xylanase — 23 U/mL and cellulase — 17 U/mL [19], have been documented in literature. However, waste materials were not employed as substrates in the study.

3.4. Statistical optimization of lipase production and oil biodegradation

The experimental design set-up and resulting responses R1 and R2 are given in Table 1. Design matrix evaluation for the response surface quadratic model showed 9 degrees of freedom (df) for the model, 3 for the lack of fit, and 4 for pure error. This ensures a valid lack of fit test, as fewer df will lead to a test that may not detect lack of fit. Standard errors were found to be similar within a type of coefficient (0.35 for linear coefficients, 0.50 for squared coefficients and 0.49 for interaction coefficients).

ANOVA for the response surface quadratic models showed model F-values of 55.54 (R1) and 41.78 (R2), which imply that the models are significant. There is only a 0.01% chance that F-values these high could occur due to noise. Values of Prob > F less than 0.0500 indicate that the model terms are significant. In this case, A, B, A^2, C^2 (R1) and A, B, C, A^2 (R2) were significant model terms. Values greater than 0.1000 indicate that the model terms are not significant. In general, larger magnitudes of t, F and smaller p values, indicate that the corresponding coefficient terms are significant. The R^2 value gives a measure of how much variability in the observed response can be explained by the experimental parameters and their interactions. The closer the value of R^2 is to 1, the better the correlation between the experimental and predicted values and the better the model predicts the response. The R^2 values for the quadratic models were 0.9862 (R1) and 0.9817 (R2). Adequate precision measures the signal to noise ratio and a ratio greater than 4 is desirable. The obtained ratios of 24.841 (R1) and 21.34 (R2) indicate adequate signals and the models can be used to navigate the design space.

The final Equations terms of coded factors are:

$$\text{Lipase activity} = 18.00 + 6.38 * A + 1.88 * B + 0.25 * C + 1.00 * AB - 0.25 * AC - 0.75 * BC - 1.25 * A2 - 0.75 * B2 - 1.00 * C2 \quad \text{[Equation 2]}$$

$$\text{Oil removal} = 88.00 + 7.88 * A + 2.50 * B + 1.38 * C - 0.022 * AB - 0.75 * AC - 0.011 * BC - 2.38 * A2 - 1.12 * B2 - 1.38. * C2. \quad \text{[Equation 3]}$$

These equations can be used to make predictions about the responses, for given levels of each of the factors. They are also useful for identifying the relative impacts of the factors by comparing the factor coefficients.

The contour plots are a way of expressing the regression equation graphically. They depict the interactions among the variables and are used to determine the optimum concentrations of each factor for good response. The contour plots for the interactions between CMW and peptone (A–B), peptone and yeast extract (B–C), and CMW and yeast extract (A–C) are depicted in Fig. 5 for R1 as well as for R2.

Validation experiments were also performed in order to determine the correctness of the model (Table 2). Five different combinations of media constituents specified by the model were used and the results obtained were compared with the predicted values, which pointed to a sound agreement between the observed and predicted values, thus confirming the validity and precision of the model.

By applying the Box Behnken Design of RSM, a maximum lipase activity of 26.37 U/mL was obtained from run 11 with 20 g/L CMW, 5 g/L peptone, and 0.55 g/L yeast extract. This is a 1.44-fold increase in

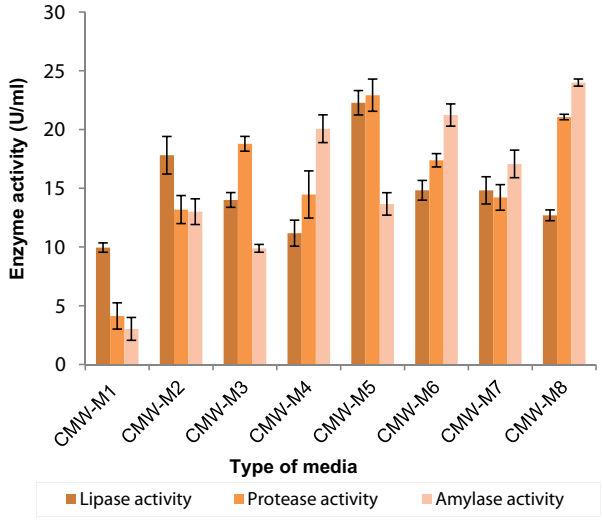

Fig. 4. Extracellular enzymatic activities of *S. pasteuri* in CMW based media. CMW-2: yeast extract alone, CMW-3: peptone alone, CMW-4: glucose/starch alone, CMW-5: yeast extract + peptone, CMW-6: peptone + glucose/starch, CMW-7: yeast extract + glucose/starch, CMW-8: yeast extract + peptone + glucose/starch. Media for amylolytic activity contained starch instead of glucose.

Table 1
Box Behnken design of RSM.

Standard	Run	Factor A[a]	Factor B[a]	Factor C[a]	R1[a]	R2[a]
17	1	12.5	2.75	0.55	18.02	88.02
7	2	5	2.75	1	11.12	80.23
10	3	12.5	5	0.1	19.09	87.98
3	4	5	5	0.55	10.14	78.91
16	5	12.5	2.75	0.55	18.39	88.53
13	6	12.5	2.75	0.55	18.51	88.12
2	7	20	0.5	0.55	20.07	91.56
5	8	5	2.75	0.1	9.72	75.35
9	9	12.5	0.5	0.1	14.45	82.63
15	10	12.5	2.75	0.55	18.71	88.82
4	11	20	5	0.55	26.37	96.03
11	12	12.5	0.5	1	15.81	84.21
8	13	20	2.75	1	22.13	92.34
12	14	12.5	5	1	17.35	89.31
6	15	20	2.75	0.1	21.38	90.87
14	16	12.5	2.75	0.55	18.61	88.12
1	17	5	0.5	0.55	8.52	73.16

[a] Factor A: CMW (g/L); Factor B: peptone (g/L); Factor C: yeast extract (g/L); R1: lipase activity (U/mL); R2: oil removal (%).

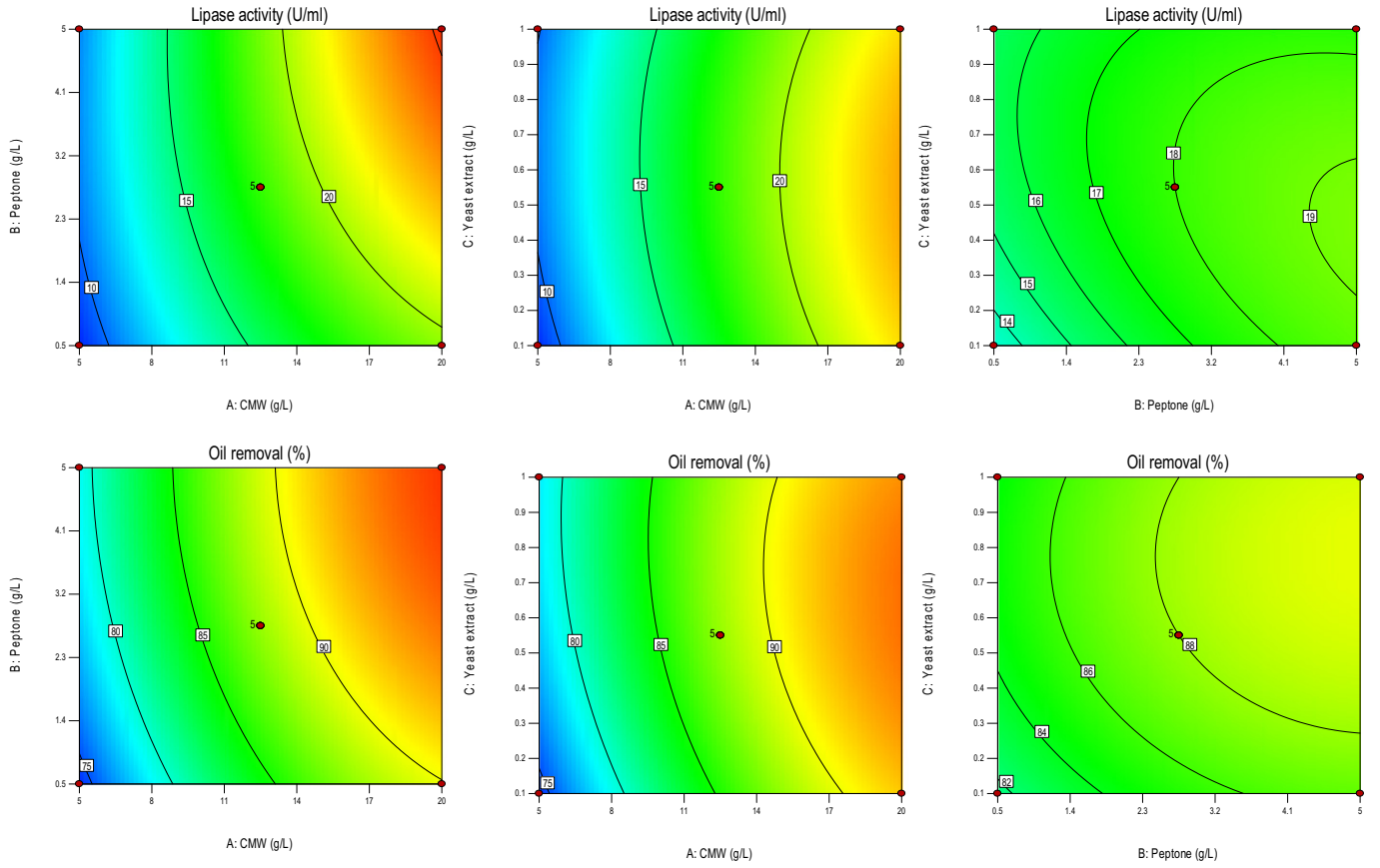

Fig. 5. Plots depicting the interactions between different variables (CMW, peptone and yeast extract) on response 1 (Lipase activity) and response 2 (oil removal).

comparison with the pre-optimization lipase activity of 18.23 U/mL. Thus, applying the statistical model has enhanced the enzyme production substantially. Moreover, 96.03% of the initial oil waste present in the medium was also biodegraded. Earlier, only 68.8% of the CMW was biodegraded when it was present at an initial concentration of 20 g/L, which means that a 1.41 fold increase in biodegradation has been achieved. This may be due to the optimal levels of peptone and yeast extract present in the medium that were conducive for growth and lipase production.

In general, lipase production from fungal species has been extensively optimized using statistical experimental design [20,21,22,23]. Such studies have also been carried out with bacteria. For instance, D-optimal design has been applied to optimize lipase production from *Geobacillus thermoleovorans* [24]. Production of extracellular lipase from *Stenotrophomonas maltophilia* CCR11 has also been optimized using statistical design [25]. Another work has utilized response surface methodology to improve lipase production from *Burkholderia* sp. C20 [26]. Alkaline lipase production from *Burkholderia multivorans* has also been similarly optimized [27]. However, the key difference is that, in our work, the enzyme production has been optimized using a cheap substrate, the oil mill waste. Moreover, biodegradation of that oil waste present in the medium, as a result of lipolytic activity has also been monitored. Besides these staple differences and the resultant benefits, a novel lipase producing strain, *S. pasteuri*, with hitherto unreported lipase activity and oil bioremediation capabilities has been exploited to perform the optimization experiments.

3.5. Lipase purification

The fractions eluted from Phenyl Sepharose CL-4B column were subjected to TBA plate assay (results not shown), in which 20 μL of fractions was added to the wells and incubated at 37°C for 2 d. The active fractions were then pooled and loaded onto the Phosphocellulose PC-11 column, eluted out, and activity was checked. Based on the results, active fractions were made into 4 pools and kept for dialysis with 50% glycerol. The electrophoretic motilities of all these fractions, along with $(NH_4)_2SO_4$ precipitated proteins are collectively depicted in Fig. 6. From the gel picture, it could be seen that the monomeric lipase band falls between the 45 and 66 kDa markers and could be concluded to be around 56 kDa. A band of around 45 kDa has been reported for *S. aureus* lipase [28]. A recombinant *S. xylosus* lipase, expressed in *Escherichia coli* has shown a band at around 43 kDa [29].

Purification table was constructed based on the enzyme activity and protein concentration obtained in each step (Table 3). Phenyl Sepharose CL-4B column resulted in a 1.98-fold purification of the enzyme and a yield of 0.63%. Phosphocellulose PC-11 column gave a far better 9.02-fold purification of the enzyme and a yield of 0.76%. In other such studies, nickel metal affinity chromatography had facilitated 8.8-fold purification of *S. xylosus* lipase [29]. 40% recovery has been reported for *S. warneri* lipase in hydroxyapatite column [17].

Table 2
Validation of the experimental model.

Run	Factor A[a]	Factor B[a]	Factor C[a]	R1		R2	
				Experimental	Predicted	Experimental	Predicted
1	5	2.75	0.1	9.72	8.87	75.35	74.25
2	12.5	0.5	0.1	14.45	13.37	82.63	81.62
3	12.5	2.75	0.55	18.71	18.00	88.82	88.00
4	20	5	0.55	26.37	25.25	96.03	94.87
5	12.5	0.5	1	15.81	15.37	84.21	84.37

[a] Factor A: CMW (g/L); Factor B: peptone (g/L); Factor C: yeast extract (g/L); R1: lipase activity (U/mL); R2: oil removal (%).

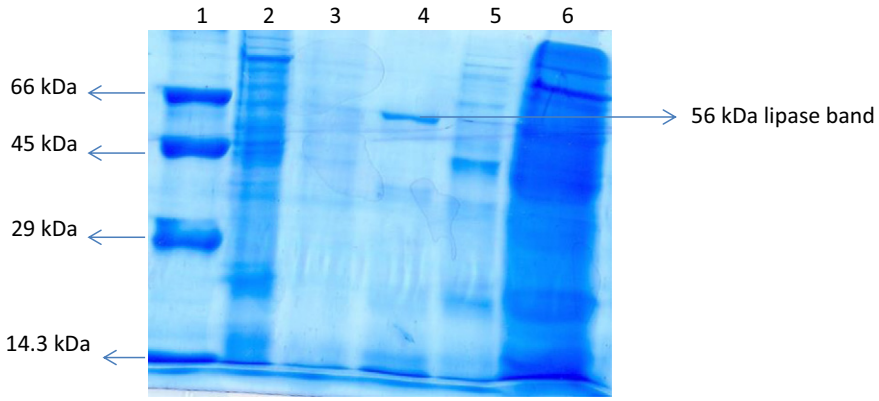

Fig. 6. SDS-PAGE of lipase. Lane 1 — marker; lane 2 — CL 4B pool 1; lane 3 — CL 4B zero salt pool, lane 4 — PC 11 second pool; lane 5 — PC 11 first pool, lane 6 — $(NH4)_2SO_4$ 75% cut.

3.6. Characterization studies

3.6.1. Effect of pH

Lipase activity increased with increasing pH and peaked at pH 9 (25.21 U/mL), after which it gradually dropped (Fig. 7). Based on this observation, it could be inferred that the enzyme is alkaliphilic in nature. Such preference for alkaline conditions is seen in most *Staphylococcal* lipases [30]. The enzyme was most stable in buffers of pH 5–9. Moreover, it was observed to be quite resistant to alkali denaturation, retaining up to 63% of its activity even after pre-incubation in highly alkaline buffers (Fig. 7). Similar results have been reported in literature. For instance, *S. xylosus* lipases wt-SXL2 and r-SXL2 were demonstrated to remain active at alkaline pH ranging from 8 to 9.5 and their maximal activities were measured at pH 8.5 [15].

3.6.2. Effect of temperature

The enzyme activity increased with increasing temperature and peaked at 50°C (29.9 U/mL). Thereafter it dropped to 18.31 U/mL at 60°C, and plunged to negligible levels at still higher temperatures (Fig. 8). The stability of COM-4A lipase was maintained upon pre-incubation at temperatures up to 60°C (Fig. 8). Exposure to 70°C or higher, caused thermal denaturation of the lipase. These results reveal that the enzyme is moderately thermophilic in nature. Such high temperature tolerance has also been reported for *S. xylosus* lipases, which retained 90% of their initial activities after 60 min incubation at 55°C [15]. Enzyme stability at high pH and temperatures is a much needed criterion for a variety of applications such as biodiesel production, biopolymer synthesis and use in detergent formulations, to name a few [31]. Since the COM-4A lipase meets this requirement, it could be exploited for such applications.

Moreover, maximum oil biodegradation was also achieved under similar conditions of pH and temperature, which substantiate the major role played by this enzyme in lipid hydrolysis.

3.6.3. Effect of metal ions, detergents and organic solvents

Lipase activity was found to be significantly enhanced in the presence of Ca^{2+} ions (127%) and to a lesser extent in the presence of K^{+} ions (112%). Cu^{2+}, Mg^{2+}, and Fe^{3+} inhibited the enzyme activity to various degrees, while Ag^{2+} (11%) resulted in drastic activity inhibition (Fig. 9). Ca^{2+}, Na^{+}, and Cu^{2+} ions have been reported to enhance the activity (14–22% increase in activity) of *S. aureus* lipase at 1 mM concentration [14]. Other works also point towards an enhancement of lipase activity especially in the presence of Ca^{2+}. In *Staphylococcus hyicus*, this has been attributed to the presence of a Ca^{2+} binding site which is formed by two conserved aspartic acid residues near the active-site, and the binding of the Ca^{2+} to this site dramatically enhanced the lipase activity [32].

Stimulation as well as inhibition of enzyme activity in the presence of amphipathic detergent molecules has been documented. Certain lipases have been shown to be capable of acting only in the presence of surfactants, as they increase the amount of oil–water interface available for the lipase to act, by emulsifying the lipidic substrate. However, the inhibitory effects of detergents on lipase have also been reported in certain other instances [33,34,35]. When the effect of detergents on COM-4A lipase was investigated, the non-ionic detergents triton-X 100 and tween-20 boosted its activity. Contrary to our observation, the inhibitory effect of triton X-100 on *Staphylococcus* lipase has been recorded by other investigators [36]. Our enzyme was also tolerant to the presence of bile salts taurocholate and deoxycholate, retaining a substantial amount of its activity (92 and 81%, respectively). Similar results have been obtained by other researchers [16]. However, the synthetic anionic detergent SDS and the cationic detergent CTAB, remarkably affected enzyme stability (Fig. 9).

Stability in the presence of organic solvents is pertinent if the enzyme is to be used for catalyzing synthetic reactions in non-aqueous milieu and optical resolution of chiral compounds [37]. In COM-4A, polar organic solvents like ethanol, methanol and isopropanol had little inhibitory effects on lipase stability. The presence of non-polar solvents like n-hexane, toluene and chloroform inhibited its activity to a greater extent, but not altogether, as a minimum of 55% residual activity was witnessed even in the extreme case (Fig. 9). *S. aureus* lipase has been shown to retain ≥80% activity after incubation with ethanol, DMSO, methanol, isopropanol, toluene, and ethylene glycol [14]. Reports of lipase remaining stable in the presence of water miscible organic solvents are scanty. This is due to the tendency of polar solvents to strip off the much needed water

Table 3
Purification table for COM-4A lipase.

S. No	Sample	Total activity (U)	Protein recovery (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
1	Crude	1900	60	31.6	1.0	100
2	75% $(NH_4)_2SO_4$ cut	852	40	21.3	0.67	44.84
3	Phenyl Sepharose CL-4B	12	0.192	62.5	1.98	0.63
4	Phosphocellulose PC-11	14.4	0.0495	285	9.02	0.76

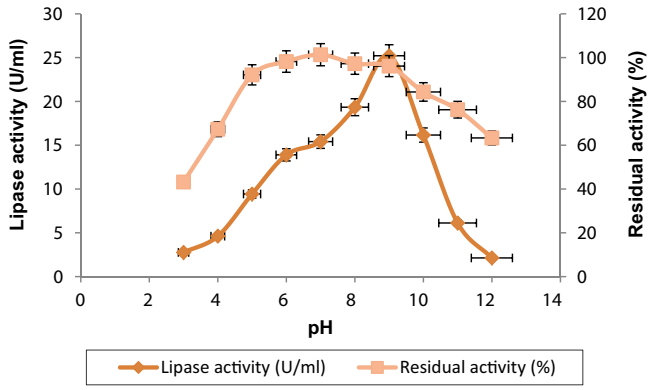

Fig. 7. Effect of pH on lipase activity and stability.

molecules from the active site of the enzyme. However, in certain cases, it is possible that a thin layer of water molecules remains tightly bound to the enzyme along its hydrophilic surfaces and acts as a protective sheath [38].

4. Conclusions

The findings from the present investigation suggest that *S. pasteuri* strain COM-4A and the lipase produced by it are quite promising and warrant further research. This is more so because *S. pasteuri* is a species with not well-documented lipase activity. The isolate has desirable features that could be favorably exploited for the treatment of wastewaters high in fat and oil contents. The combined hydrolytic activities of lipase, protease and amylase possessed by *S. pasteuri* could prove to be beneficial for bringing down the overall organic load of such wastewaters. Moreover, pretreatment of high lipid content wastewaters using hydrolytic enzymes is a proven pre-treatment strategy that makes them more amenable to anaerobic digestion. The process optimizations carried out as part of this study could serve as valuable tools in augmenting the isolate's performance in such bioremediation scenarios. Purification and characterization of lipase from COM-4A has further revealed its industrially useful properties such as stability at high pH values, high temperatures, and in the presence of certain organic solvents and detergents. Such traits mean that the use of this enzyme could be extended to diversified industrial sectors as well, such as in detergent formulations and in biodiesel production, thereby giving it an edge over other lipases.

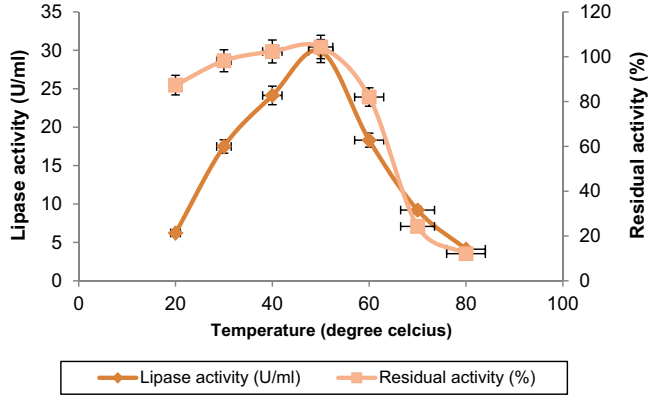

Fig. 8. Effect of temperature on lipase activity and stability.

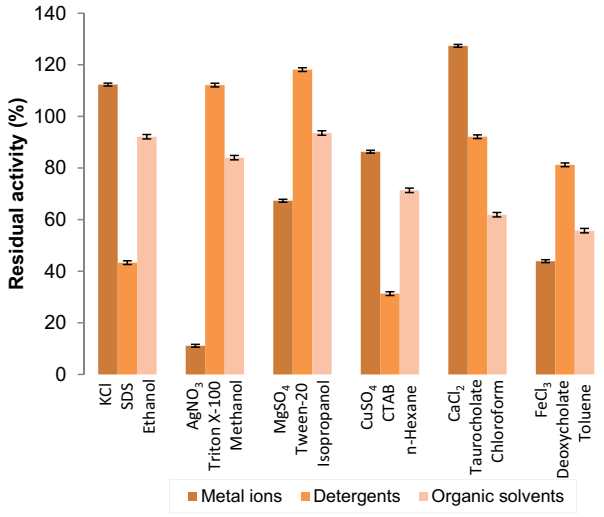

Fig. 9. Effect of metal ions, detergents and organic solvents on lipase stability.

Acknowledgments

The authors are thankful to the management of Kumaraguru College of Technology, Coimbatore, India, for providing the laboratory facilities to carry out this work.

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