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Lipase from marine *Aspergillus awamori* BTMFW032: Production, partial purification and application in oil effluent treatment

Research Paper

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Marine fungus BTMFW032, isolated from seawater and identified as *Aspergillus awamori*, was observed to produce an extracellular lipase, which could reduce 92% fat and oil content in the effluent laden with oil. In this study, medium for lipase production under submerged fermentation was optimized statistically employing response surface method toward maximal enzyme production. Medium with soyabean meal-0.77% (w/v); $(NH_4)_2SO_4$-0.1 M; KH_2PO_4-0.05 M; rice bran oil-2% (v/v); $CaCl_2$-0.05 M; PEG 6000-0.05% (w/v); NaCl-1% (w/v); inoculum-1% (v/v); pH 3.0; incubation temperature 35 °C and incubation period-five days were identified as optimal conditions for maximal lipase production. The time course experiment under optimized condition, after statistical modeling, indicated that enzyme production commenced after 36 hours of incubation and reached a maximum after 96 hours (495.0 U/ml), whereas maximal specific activity of enzyme was recorded at 108 hours (1164.63 U/mg protein). After optimization an overall 4.6-fold increase in lipase production was achieved. Partial purification by $(NH_4)_2SO_4$ precipitation and ion exchange chromatography resulted in 33.7% final yield. The lipase was noted to have a molecular mass of 90 kDa and optimal activity at pH 7 and 40 °C. Results indicated the scope for potential application of this marine fungal lipase in bioremediation.

Introduction

Lipases (triacylglycerol acylhydrolase EC 3.1.1.3) are serine hydrolases that catalyze both the hydrolysis and the synthesis of esters from glycerol and long chain fatty acids. They are considered as the third largest enzyme group, after proteases and carbohydrases, based on their market value. Owing to a large potential for lipases in industrial applications such as additives in foods, pharmaceuticals, medical assay, cosmetics, leather, dairy industry, fine chemicals, detergents, paper manufacture and waste-water treatment [1], lipases from microorganisms [2] have recently drawn greater attention of investigators and industries in spite of their occurrences in plants [3,4], and animals [5,6]. Among the various microorganisms recognized as source of lipases, filamentous fungi are considered as the best source of extracellular lipase for large scale production by industries. Particularly, species belonging to *Rhizopus*, *Mucor*, *Geotrichum*, *Penicillium* and *Aspergillus* are widely recognized as best source of lipase [7–9]. Among them several species of *Aspergillus*, isolated from terrestrial sources, have been reported to produce lipase [7,10] with remarkable properties suitable for biotechnological applications. Although microbial lipases is one among the major subject of intensive research on industrial enzymes at global level, marine microbial lipases remains yet unexploited.

Medium components are known to greatly affect the production of metabolites [11] by microorganisms and hence it is imperative to evaluate the nutritional requirements toward enhancing productivity. The conventional approach 'one factor per trial'

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employed for medium optimization is time-consuming, expensive and often impractical when large number of variables needs to be investigated. Whereas, statistical optimization facilitates drawing a deep insight on the interactive effect of various parameters that influence enzyme production. Among the statistical methods available, factorial design and response surface analysis are recognized as important tools that enable accurate determination of optimal process conditions. Moreover statistical approach employing Plackett–Burman (PB) design and response surface methodology (RSM) have gained, recently, greater and wide application in various studies including medium optimization and understanding of the interactions among various physico-chemical parameters using a minimum number of experiments [12–14].

The growing concern for waste and waste-water management toward conservation of environment and sustainable development has led to the adoption of several bioremediation techniques. In this context, the use of extracellular enzymes such as esterases, lipases and proteases that hydrolyze ester bonds has assumed greater attention as a means for enhancing bioremediation of effluents enriched with fats, oils and proteins that are discharged by dairy industry, slaughter houses, restaurants, ayurvedic hospitals and health resorts. A large number of pretreatment systems (tilted plate separators, grease-trap, dissolved air flotation systems and physico–chemical treatment) are employed to remove fats and oil from the wastewater before the main treatment process which is often a biological treatment [15,16]. However, there is a need for improving the existing wastewater treatment processes by complimenting with enzyme catalysts which could facilitate easy and rapid reduction of complex substances such as fats and oil. In this context microbial lipases could have an impact on reducing the fats and oil contents in such effluents. Esterases also play an important role in the biodegradation of many natural substances in wastewater including lipids, and many synthetic chemicals by hydrolyzing short chain esters, whereas the longer chain esters could be hydrolyzed by a more limited set of enzymes, such as lipases [17]. Available research reports and patents that describe the use of lipases for the biological treatment of effluents with high fat and oil concentrations have been reviewed recently [18].

Considering the importance of lipase for several application and need for exploration of new and additional source for industrial production of lipases, we made an attempt to explore the prospect of deriving potential fungi from marine environment capable of producing lipase with desirable properties for industrial applications. Herein we report the lipase production by a fungal isolate obtained from seawater, media optimization toward maximal enzyme production under submerged fermentation (SmF), purification of enzyme, and its probable application in the treatment of oil laden effluents.

Materials and methods

Microorganism and culture maintenance

The fungal strain BTMFW032 isolated from Arabian Seawater of Kerala coast, India, and available as a stock culture at the Microbial Technology Laboratory of the Department of Biotechnology, Cochin University of Science & Technology, India, was used in the present study. Isolation of the selected fungal isolate was performed as described below.

Seawater samples were collected from a depth of 2 m using sterile water sampler and transferred to sterile bottles and stored in ice. They were immediately transferred to the laboratory, serially diluted, and plated in malt extract (ME) fungal agar medium containing 2.5% NaCl. After eight days of incubation at 28 °C the colonies obtained were subcultured on the same ME agar medium and were screened for lipase production using Rhodamine B-olive oil agar plates [19]. From among the potential lipase producers noted, fungal isolate BTMFW032 was selected based on its ability to utilize different oils as substrate and produce maximum lipase enzyme compared to other fungal isolates. The organism was maintained at 4 °C on ME fungal agar medium with the following composition (/L): 10 g malt extract, 1 g NH_2NO_3, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 1 g yeast extract, 25 g NaCl, 20 ml Tween 80, 20 g agar and pH 5.5 and subcultured fortnightly.

Identification of the fungal strain

The fungal isolate BTMFW032 was grown in ME agar medium and the conidial characteristics were observed by slide culture technique [20] using a phase contrast microscope (Nikon, Japan). Further, the conidia were coated with platinum and their structures were observed using scanning electron microscope (SEM, JEOL Model JSM - 6390LV, Japan) at 15 kV and 200–2000× magnification.

In addition to recording morphological characteristics of the fungal isolate BTMFW032, molecular systematic approach was also employed using 28S rRNA gene sequence of the fungal isolate according to the method of O'Donnell [21]. A portion of the 28S rRNA gene was amplified from the genomic DNA by polymerase chain reaction (PCR) using the specific primers NL1F 5′-GCATATCAATAAGCGGAGGAAAAG-3′ and NL4R 5′-GGTCCGTGTTTCAAGACGG-3′. Nucleotide sequences of the PCR amplicon were determined using ABI Prism 310 genetic analyzer using the big dye Terminator kit (Applied Biosystems). Identity of the sequence was established by comparing with the gene sequences available in the database using BLAST software [22] (http://blast.ncbi.nlm.nih.gov/). Later a homology search was performed and based on the results a Phylogenetic tree was constructed using the neighbor joining method implemented in Multiple Sequence Alignment-ClustalW [23] (http://align.genome.jp).

Submerged fermentation and lipase recovery

Lipase production by the fungal isolate BTMFW032, which was later identified as *Aspergillus awamori* was carried out, initially, in 50 ml of ME medium taken in 250 ml Erlenmeyer flask and supplemented with rice bran oil 2% (v/v) as substrate. Oil was sterilized separately in a hot air oven for 2 hours at 160 °C, cooled to room temperature (RT, 28 ± 2 °C), and then added to the autoclaved medium under stirring condition to effect good mixing and attain homogeneity of the medium. The spore inoculum was prepared using a freshly raised ten days old ME agar slant culture and by dispersing the spores in 0.1% Tween 80 (v/v) prepared in physiological saline. Two milliliter of the spore suspension (7×10^7 cfu/ml), was used as inoculum unless otherwise mentioned. The inoculated medium was incubated at RT (28 ± 2 °C) and at 150 rpm in an environmental shaker (Orbitek, Scigenics India) for five days (arbitrarily selected). After fermentation the mycelial biomass was separated by filtration through Whatman

filter paper No.1 and the culture supernatant (considered as crude enzyme extract) was assayed for lipase activity.

Assay of lipase activity

Lipase activity was assayed spectrophotometrically using pNP Caprylate (pNPC8) as substrate (Fluka-Chemie, Germany), according to the modified method of Prim *et al.* [24] in a microtiter plate and the absorbance was measured at 415 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of pNP per minute under the assay conditions. All the media components and chemicals used in this study were of highest purity grade available commercially from Hi-media, Sisco Research Laboratories Ltd., India, and Sigma–Aldrich, USA.

Protease assay and protein estimation

Protease activity was determined by caseinolytic method [25] with minor modification. Hammerstein casein was used as substrate for the assay, and the increase in absorbance was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme that liberated 1 μg of tyrosine per milliliter of the reaction mixture per minute under the assay conditions (pH 7, 37 °C). Protein content of the sample was estimated according to the method of Lowry *et al.* [26] using bovine serum albumin (BSA) as the standard.

Statistical optimization of medium components for lipase production

During the course of our earlier studies, it was observed that the marine *A. awamori* BTMFW032 produced lipase under SmF. Hence, statistical optimization of medium components for maximum lipase production was carried out in two stages employing Plackett–Burman (PB) design followed by RSM using Box–Behnken design. Design-Expert (Version 6.0, Stat Ease Inc., Minneapolis, USA) software was used for experimental design and interpretations.

Plackett–Burman experimental design

Plackett–Burman (PB) design [27], a two-level factorial design method, was used to identify the medium components that have significant effects on lipase production under SmF. Eleven factors which showed considerable influence on lipase production during the course of optimization studies conducted earlier employing 'one factor per trial' (data not shown) were used for the study. A set of 12 experiments was used to determine the relative effect of 11 factors, comprising 4 physical factors (pH, temperature, incubation period and inoculum size) and 7 nutritional factors (soyabean meal, rice bran oil, $(NH_4)_2SO_4$, NaCl, $CaCl_2$, KH_2PO_4 and PEG 6000). All factors were investigated at two widely spaced levels designated −1 (low level) and +1 (high level). The experimental responses were analyzed by the method of least squares to fit the first-order model, given as Eq. (1):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i, \quad (1)$$

where Y is the predicted response (lipase yield), β_0 is the model intercept, β_i is the linear coefficient, X_i is the coded level of the independent variable, and k is the number of involved variables. All the trials were carried out in triplicate, and the average lipase yield for each trial was used as the response variable. Statistical significance of the model equation was determined by Fischers test and the proportion of variance explained in the model was presented by the multiple coefficient of determination, R^2 value. Design Expert software was used for the matrix design and interpretations.

Box–Behnken experimental design

The levels of the significant parameters and the interaction effects between various medium constituents which influence the lipase production significantly were analyzed and optimized by Box–Behnken design [28]. Each independent variable was studied at three different levels (low, medium and high, coded as −1, 0 and +1, respectively). All the variables were taken at a central coded value, considered as zero. A design model with 46 experiments in two blocks of 23 cases was used. All the experiments were done in triplicate and the average of lipase production obtained was taken as the dependent variable or response (Y). The second order polynomial coefficients were calculated and analyzed using the 'Design Expert' software. To correlate the response variable (i.e., lipase yield) to the independent variables, the yield was fitted according to the following second-order polynomial model Eq. (2):

$$Y = \beta_0 + \sum_{i=1}^{5} \beta_i X_i + \sum_{i=1}^{5} \beta_{ii} {X_i}^2 + \sum_{i=1}^{5} \beta_{ij} X_i X_j \quad (2)$$

where Y represents the response variable (lipase yield), β_0 is the interception coefficient, β_i is the coefficient of the linear effect, β_{ii} is the coefficient of quadratic effect and βij is the coefficient of interaction effect when $i < j$. Five significant variables (soyabean meal, $(NH_4)_2SO_4$, KH_2PO_4, temperature and incubation period) were used in Box–Behnken design.

A multiple regression analysis of the obtained data was carried out to define the response in terms of the independent variables. The goodness-of-fit of the regression model was obtained from the multiple correlation coefficients R and by the coefficient of determination R^2. The statistical significance of the model was determined by the application of Fischer's test. Three dimensional response surface graphs were obtained to understand the effect of variables individually and in combination, and to determine their optimum levels for maximum lipase production.

Validation of the model

To validate the response surface model, a random set of seven experimental combinations were conducted according to the conditions predicted by the model and a time course study was also conducted with the optimized parameters in shaken flasks in triplicate.

Purification of lipase

The crude enzyme extract obtained after SmF was purified employing $(NH_4)_2SO_4$ precipitation, followed by dialysis and ion-exchange chromatography. Various concentrations of $(NH_4)_2SO_4$ (20, 40, 60, 80 and 90%) were used to standardize the precipitation of lipase from the fermented broth. The precipitated protein was resuspended in 0.1 M phosphate buffer (pH 7), dialysed against 0.01 M solution of the same buffer for 24 hours, at 4 °C, with six changes of buffer and assayed for lipase activity and protein content.

Further purification was done by ion-exchange chromatography using DEAE cellulose. The pH at which the enzyme binds at its

maximum to DEAE-cellulose was standardized to pH 7.5. Dialysed sample, with a protein content of 1.41 mg/ml, was applied to DEAE-cellulose column (XK16/26-Amersham Biosciences) that had been pre-equilibrated with phosphate buffer (pH 7.5, 0.01 M). Unbound proteins were washed with the same buffer and stepwise elution was done at a flow rate of 2 ml/min using 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl in the same buffer. Fractions (5 ml) were collected and protein content was estimated by measuring the absorbance at 280 nm. Peak fractions from the column were pooled and assayed for lipase activity and protein content.

Characterization of the enzyme

SDS-PAGE and zymography

Samples precipitated with $(NH_4)_2SO_4$ and active fractions collected after ion exchange chromatography, were electrophoresed by Native-PAGE and SDS-PAGE in a 10% polyacrylamide gel according to the method of Laemmlli [29]. One milliliter aliquots of each of the samples were lyophilized and resuspended in 0.1 ml of sample buffer (0.0625 M Tris–HCl, 2% SDS, 10% sucrose, pH 6.8) and subjected to electrophoresis. For the detection of proteins, gels were stained with coomassie brilliant blue R250 after electrophoresis. Low molecular weight markers of Amersham Pharmacia was used as molecular mass standard and molecular weight of lipase was determined using Quantity One® Software of Biorad.

Lipase activity of the enzyme protein band was confirmed by zymogram analysis [30] using the fluorogenic substrate 4-methylumbelliferyl butyrate (MUF-butyrate) (Sigma, St. Louis, USA). Ammonium sulfate precipitated (40–90%) fraction and active fractions pooled from ion exchange chromatography were lyophilized and resuspended in 0.1 ml of sample buffer and subjected to electrophoresis in a 10% polyacrylamide gel at 4 °C. After electrophoresis, SDS was removed by soaking the gel in 2.5% (v/v) Triton X-100 for 30 min at RT (28 ± 2 °C) followed by brief washing in 50 mM phosphate buffer, pH 7.0. Further, the gel was covered by a solution of 100 μM MUF-butyrate prepared by diluting in 50 mM phosphate buffer from a stock of 25 mM MUF-butyrate in Methyl cellosolve (Sisco Research Laboratories Pvt. Ltd., India). Lipase activity for the bands was visualized by the presence of fluorescence under UV light.

Gel filtration chromatography

Gel filtration chromatography was conducted using the $(NH_4)_2SO_4$ precipitated fraction, to confirm the presence of different lipases in the sample. Sephadex G-100 (Sigma-Aldrich), used as the column matrix, was suspended in distilled water and allowed to hydrate for 3 hours at 100 °C in a water bath, and the fine particles were removed by decantation. The hydrated gel suspension was then degassed under vacuum to remove the air bubbles. The gel suspension was then carefully poured into the column (Amersham Biosciences XK26/70 column) without trapping air bubbles and then allowed to settle under gravity while maintaining a slow flow rate through the column. Later, the column was stabilized by allowing two times the bed volumes of eluent (0.1 M phosphate buffer, pH 7.5) to pass through the column bed in descending eluent flow. An aliquot of 2 ml of the sample, obtained after $(NH_4)_2SO_4$ precipitation, with a protein content of 1.41 mg/ml, was then applied to the column. After complete entry of the sample into the column the proteins were eluted using 0.1 M phosphate buffer of pH 7.5, at a flow rate of 1 ml/min. Eluent fractions (1 ml) were collected and their protein contents were estimated by measuring the absorbance at 280 nm in a UV–visible spectrophotometer (Shimadzu, Japan). Peak fractions from the column were pooled and assayed for the lipase activity and the protein content. Molecular mass was calculated from the calibration curve prepared using the calibration kit proteins (Amersham Pharmacia) and by plotting the semilogarithmic graph for the K_{av} versus molecular weight of the standard proteins.

Determination of optimal pH and temperature of purified enzyme

Optimum pH for maximal activity of the purified enzyme was determined by checking the enzyme activity in different buffer systems of pH 2–13 which included, 0.05 M buffer solutions of HCl–KCl (pH 2), citrate–phosphate (pH 3–6), phosphate (pH 7), Tris–HCl (pH 8–9), carbonate–bicarbonate (pH 10), and Titrisol® (pH 11, 12 and 13; Merck, Germany). Experiments were conducted using 0.2 ml of the diluted enzyme sample and pNP Palmitate (pNP Caprylate was not used because it was unstable at pH above 8) as substrate prepared in the respective buffer of each pH. The temperature optimum for maximal enzyme activity was determined by assaying the enzyme activity at different incubation temperatures (5–100 °C).

Oil effluent treatment with fungal lipase

The fungal lipase was evaluated for its potential application in the treatment of effluents laden with oil using various effluents which included simulated effluents blended with 10% each of 'used restaurant oil', 'oil from ayurvedic hospitals used for massaging and treatment', 'used fish fry oil', palm oil, coconut oil and dalda. The prepared effluent was treated with 1% (v/v) enzyme (300 U/mL) for 24 hours at 35 °C, with an agitation at 100 rpm. Simulated effluent with 1% heat inactivated enzyme was used as control. After 24 hours of incubation, fat and oil content in the treated effluent was estimated according to the AOAC official method 989.05 [31] and the percentage reduction in fat and oil was calculated and expressed.

Results and discussion

Identification of the fungus

The fungal isolate BTMFW032 formed a black colony in ME agar plate and their conidiophores observed under the microscope resembled those of *Aspergillus* sp. (Fig. 1a). Generally black conidia were reported for *A. niger* or *A. awamori*; where as those of other species of *Aspergillus* were brown, yellow, or green [32]. Further, SEM observations indicated a conidial head diameter of 250 μm and conidium diameter of 2.8 μm (Fig. 1b,c). Kanauchi *et al.* [33] reported *A. awamori* with 220 μm and 3.8 μm diameter for conidial head and conidia, respectively. The black conidial heads, fast growing colonies with biseriate sterigmata and around 2–4 μm diameter conidia differentiate *A. awamori* from the closely related species *A. niger* [34]. The fungal isolate BTMFW032 was identified as *A. awamori* Nagazawa and deposited at the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Presence of fluorescence in Rhodamine B-olive oil agar plate noted during screening of lipase production by *A. awamori* (Fig. 1d) strongly

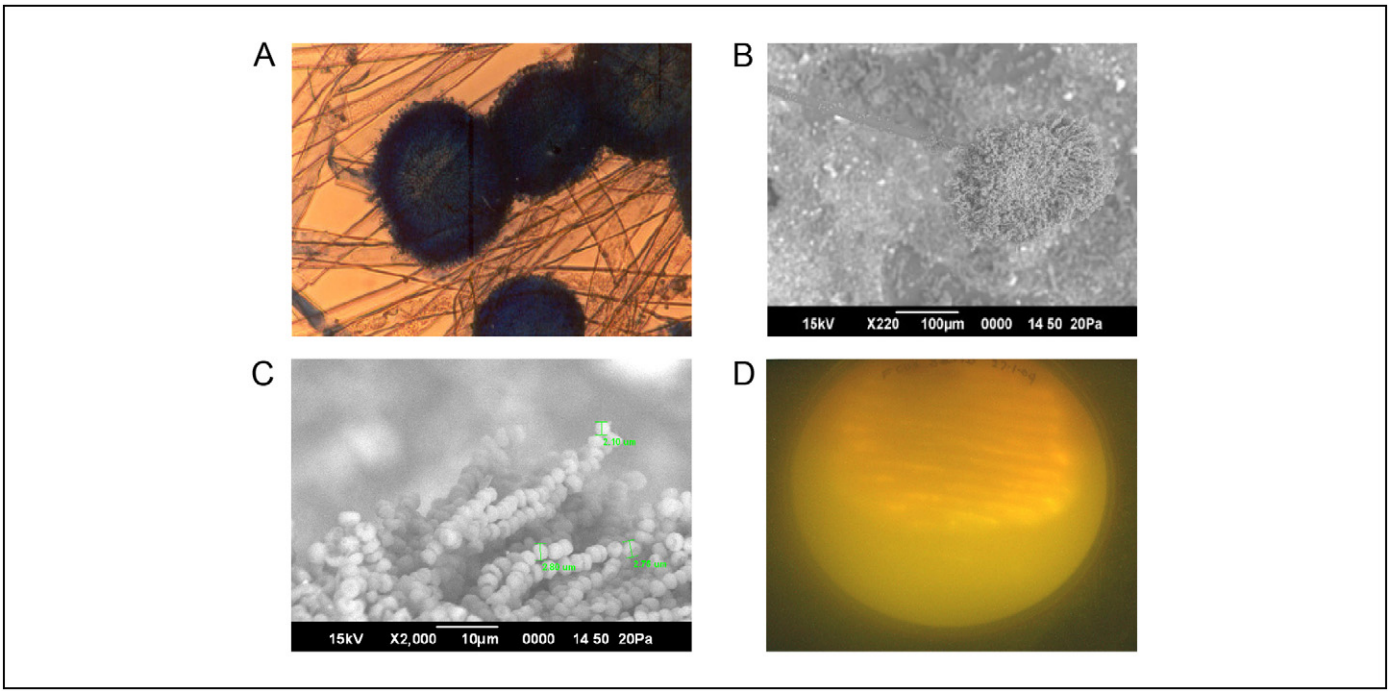

FIGURE 1

Aspergillus awamori BTMFW032: **(a)** photomicrograph of conidial head ×400 (phase contrast microscope, Nikon, Japan). **(b, c)** SEM of conidial head and conidia respectively (JEOL Model JSM - 6390LV, Japan). **(d)** Lipase activity as orange-red fluorescence upon UV irradiation in rhodamine B-olive oil agar plate.

indicated the suitability of this fungus for production of extracellular lipase.

Molecular techniques utilizing the amplification of target DNA provide alternate methods for diagnosis and identification [35]. A portion of the 28S rRNA gene was amplified from the genomic DNA using a primer pair for 28S rDNA (NL1F and NL4R) and their partial nucleotide sequence (764 bp) was submitted to GenBank with the accession number EF524198 through BankIt programme, at NCBI site. The identity of the sequence was determined by BLAST software and the results showed 91% sequence similarity with the already available sequences of *A. niger* in the GenBank. Phylogenetic tree was constructed using the NJ method, which indicated the relationship of BTMFW032 with other reported *Aspergillus* sp. (Fig. 2).

Statistical optimization of medium components for lipase production

Lipases are generally induced by lipid substrates and are thus produced with high specific activity in the presence of oil as a carbon source [36,37]. Almost all the oils used as substrates in the present study supported lipase production, although maximum enzyme production was obtained with rice bran oil (2% v/v) (data not shown). The variation in preferential utilization of specific oil as substrate for maximal enzyme synthesis may be attributed to the diversity in nutritional requirement of each species of fungi. The presence of glucose in the medium showed a negative influence (data not shown), suggesting a probable catabolite repression [9,38] in the presence of oils as carbon source.

Medium optimization by a conventional 'one factor per trial' approach does lead to a substantial increase in enzyme yield. However, this approach not only is cumbersome and time consuming, but also has the limitations of ignoring the importance of interaction of various physicochemical parameters [39]. Hence, medium that could support maximal lipase production by *A. awamori* was optimized employing statistical approach, using the factors selected after optimization studies carried out employing 'one factor per trial' method. The selected process variables were optimized statistically using PB design followed by RSM using Box Behnken design. Experiments were conducted for lipase production by *A. awamori* under SmF in shake flasks.

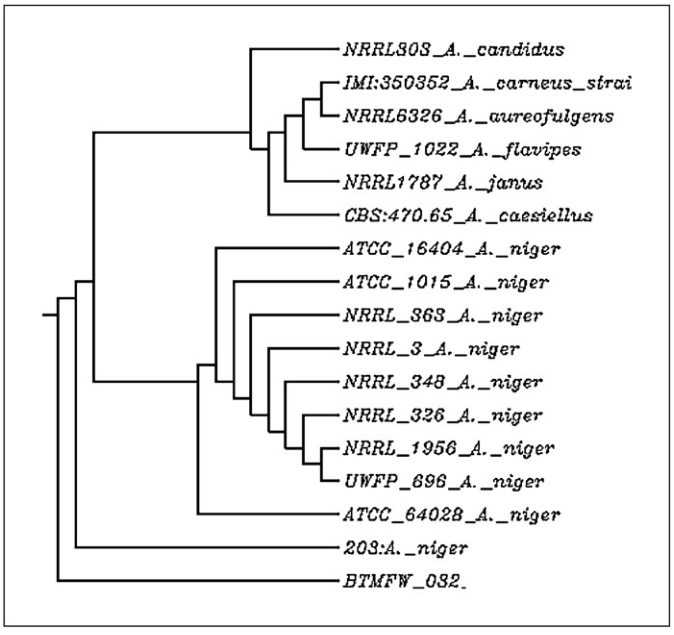

FIGURE 2

Phylogram showing the relationship between marine *A. awamori* BTMFW032 and other related species of *Aspergillus*.

TABLE 1

The matrix of the PB design experiment, together with the observed experimental data for lipase production by marine *A. awamori* BTMFW032

Run	Variables											Lipase activity (U/ml)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Y
1	(−1) 3	(+1) 5	(−1) 0.5	(+1) 0.1	(+1) 2	(−1) 2	(+1) 0.1	(+1) 0.1	(−1) 0.05	(−1) 1	(+1) 40	2.0
2	(+1) 5	(+1) 5	(−1) 0.5	(+1) 0.1	(−1) 1	(+1) 4	(−1) 0.05	(−1) 0.05	(−1) 0.05	(+1) 4	(+1) 40	39.5
3	(−1) 3	(+1) 5	(+1) 1	(+1) 0.1	(−1) 1	(+1) 4	(+1) 0.1	(−1) 0.05	(+1) 0.1	(−1) 1	(−1) 30	103.6
4	(+1) 5	(+1) 5	(+1) 1	(−1) 0.05	(−1) 1	(−1) 2	(−1) 0.05	(+1) 0.1	(+1) 0.1	(−1) 1	(+1) 40	4.0
5	(−1) 3	(−1) 3	(−1) 0.5	(−1) 0.05	(−1) 1	(−1) 2	(−1) 0.05	(−1) 0.05	(−1) 0.05	(−1) 1	(−1) 30	66.5
6	(−1) 3	(−1) 3	(+1) 1	(+1) 0.1	(+1) 2	(−1) 2	(−1) 0.05	(−1) 0.05	(+1) 0.1	(+1) 4	(+1) 40	88.4
7	(+1) 5	(−1) 3	(+1) 1	(−1) 0.05	(+1) 2	(+1) 4	(+1) 0.1	(−1) 0.05	(−1) 0.05	(−1) 1	(+1) 40	57.7
8	(+1) 5	(+1) 5	(−1) 0.5	(−1) 0.05	(+1) 2	(−1) 2	(+1) 0.1	(−1) 0.05	(+1) 0.1	(+1) 4	(−1) 30	38.5
9	(−1) 3	(−1) 3	(−1) 0.5	(−1) 0.05	(−1) 1	(+1) 4	(+1) 0.1	(+1) 0.1	(+1) 0.1	(+1) 4	(+1) 40	2.8
10	(+1) 5	(−1) 3	(−1) 0.5	(+1) 0.1	(+1) 2	(+1) 4	(−1) 0.05	(+1) 0.1	(+1) 0.1	(−1) 1	(−1) 30	3.2
11	(−1) 3	(+1) 5	(+1) 1	(−1) 0.05	(+1) 2	(+1) 4	(−1) 0.05	(+1) 0.1	(−1) 0.05	(+1) 4	(−1) 30	53.4
12	(+1) 5	(−1) 3	(+1) 1	(+1) 0.1	(−1) 1	(−1) 2	(+1) 0.1	(+1) 0.1	(−1) 0.05	(+1) 4	(−1) 30	40.8

X_1 – incubation days, X_2 – pH, X_3 – soyabean meal (%), X_4 – $(NH_4)_2SO_4$ (M), X_5 – NaCl (%), X_6 – ricebran oil (%), X_7 – $CaCl_2$ (M), X_8 – KH_2PO_4 (M), X_9 – PEG 6000 (%), X_{10} – inoculum (%), X_{11} – temperature (°C), (+1) – high level, (−1) – low level.

Screening of parameters using Plackett–Burman design

Data obtained for the studies conducted on optimization of medium for lipase production using PB design were analyzed by Design expert@ software and a first-order model (Eq. (1)) was fitted to the data obtained from the experiment. The experimental results obtained for the lipase production studies conducted with PB design are shown in Table 1. The lipase enzyme synthesized by *A. awamori* was found to vary from 2.0 to 103.6 U/ml in the 12 experiments conducted. The observations clearly indicated that there was a considerable variation in the lipase activity depending on the medium composition. The statistical analysis of the PB design showed that the variables-incubation period (X_1), soyabean meal (X_3), $(NH_4)_2SO_4$ (X_4), KH_2PO_4 (X_8) and temperature (X_{11}), have profound effect on lipase production

The statistical significance of the model equation was evaluated by the *F*-test ANOVA, which revealed that this regression is statistically significant. The results were used to fit a first order polynomial equation and the model equation for lipase yield (Y) could be written as:

$$\text{lipase activity } Y\,(\text{U/ml}) = 40.69 - 11.28X_1 + 16.61X_3 + 4.69X_4 - 25.0X_8 - 9.75X_{11}$$

The model coefficients estimated by multiple linear regressions and ANOVA showed *F*-value of 71.55 and P value of 0.0001. The value of P less than 0.05 denoted that the model terms are significant. This fit of the model was checked with the coefficient of determination R^2, which was calculated to be 0.9835, indicated that 98% of the variability in the response could be explained by the model. The closer the R^2 is to 1.0, the stronger the model and the better it predicts the response [40]. The predicted R^2 of 0.9835 was in agreement with the adjusted R^2 of 0.9698. This signified a good agreement between the experimental and predicted values for lipase production. The adequate precision which measures the signal to noise ratio was 25.578. A ratio of >4 is desirable and this model can be used to navigate the design space.

A graphical representation of the effects of individual parameters studied in PB design was presented as Pareto chart in Fig. 3. The data evidence that soyabean meal and $(NH_4)_2SO_4$ had a positive effect in enhancing enzyme production along with increase in their concentrations, while KH_2PO_4 followed by incubation period and temperature had a negative effect on enzyme production along with the increase in the level of variables.

Optimization of significant variables using response surface methodology

Box–Behnken design experiment of RSM was adopted toward selection of optimal level of the variables incubation period (X_1), soyabean meal (X_2), $(NH_4)_2SO_4$ (X_3), KH_2PO_4 (X_4) and incubation temperature (X_5) which were identified as significant based on the PB design experiment. The design matrix (Box–Behnken design) and the corresponding experimental data obtained are shown in Table 2.

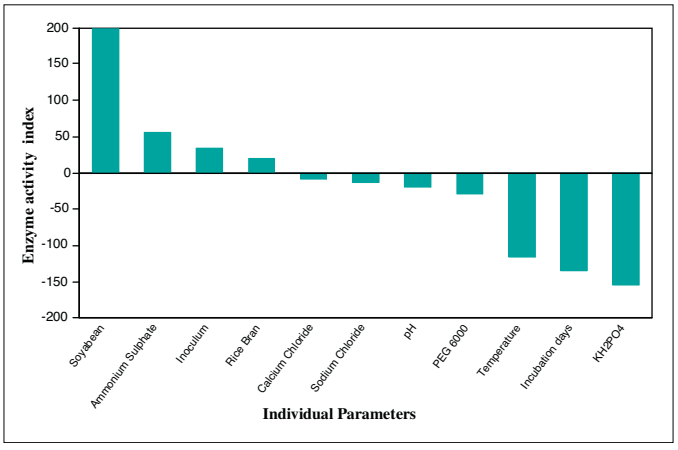

FIGURE 3

Pareto chart showing the effect of individual factors on lipase production by marine *A. awamori* BTMFW032.

TABLE 2

Effect of individual variable on lipase production by marine *A. awamori* BTMFW032 studied using Box–Behnken design experiment

Block	Run	Incubation period X_1 (days)	Soyabean meal X_2 (% w/v)	$(NH_4)_2SO_4$ X_3 (M)	KH_2PO_4 X_4 (M)	Temperature X_5 (°C)	[a]Lipase activity Y (U/ml)
1	1	(+1) 5	(−1) 0.50	(0) 0.08	(0) 0.08	(0) 35	324.75
1	2	(0) 4	(+1) 1.00	(0) 0.08	(0) 0.08	(−1) 30	193.90
1	3	(0) 4	(0) 0.75	(−1) 0.05	(−1) 0.05	(0) 35	253.96
1	4	(−1) 3	(−1) 0.50	(0) 0.08	(0) 0.08	(0) 35	130.00
1	5	(+1) 5	(0) 0.75	(+1) 0.10	(0) 0.08	(0) 35	272.95
1	6	(0) 4	(+1) 1.00	(0) 0.08	(0) 0.08	(+1) 40	404.20
1	7	(0) 4	(0) 0.75	(0) 0.08	(0) 0.08	(0) 35	401.34
1	8	(−1) 3	(0) 0.75	(−1) 0.05	(0) 0.08	(0) 35	229.50
1	9	(0) 4	(−1) 0.50	(0) 0.08	(0) 0.08	(+1) 40	338.00
1	10	(0) 4	(0) 0.75	(0) 0.08	(+1) 0.10	(+1) 40	180.18
1	11	(+1) 5	(0) 0.75	(−1) 0.05	(0) 0.08	(0) 35	278.70
1	12	(−1) 3	(+1) 1.00	(0) 0.08	(0) 0.08	(0) 35	237.50
1	13	(0) 4	(0) 0.75	(0) 0.08	(0) 0.08	(0) 35	423.70
1	14	(0) 4	(0) 0.75	(0) 0.08	(−1) 0.05	(+1) 40	403.20
1	15	(0) 4	(−1) 0.50	(0) 0.08	(0) 0.08	(−1) 30	56.90
1	16	(+1) 5	(+1) 1.00	(0) 0.08	(0) 0.08	(0) 35	462.03
1	17	(−1) 3	(0) 0.75	(+1) 0.10	(0) 0.08	(0) 35	425.78
1	18	(0) 4	(0) 0.75	(−1) 0.05	(+1) 0.10	(0) 35	283.14
1	19	(0) 4	(0) 0.75	(0) 0.08	(−1) 0.05	(−1) 30	322.39
1	20	(0) 4	(0) 0.75	(0) 0.08	(0) 0.08	(0) 35	396.73
1	21	(0) 4	(0) 0.75	(0) 0.08	(+1) 0.10	(−1) 30	64.02
1	22	(0) 4	(0) 0.75	(+1) 0.10	(+1) 0.10	(0) 35	298.69
1	23	(0) 4	(0) 0.75	(+1) 0.10	(−1) 0.05	(0) 35	394.40
2	1	(0) 4	(−1) 0.50	(−1) 0.05	(0) 0.08	(0) 35	287.00
2	2	(+1) 5	(0) 0.75	(0) 0.08	(−1) 0.05	(0) 35	478.20
2	3	(0) 4	(+1) 1.00	(0) 0.08	(+1) 0.10	(0) 35	271.12
2	4	(−1) 3	(0) 0.75	(0) 0.08	(0) 0.08	(+1) 40	56.73
2	5	(−1) 3	(0) 0.75	(0) 0.08	(−1) 0.05	(0) 35	330.75
2	6	(0) 4	(0) 0.75	(−1) 0.05	(0) 0.08	(+1) 40	338.40
2	7	(+1) 5	(0) 0.75	(0) 0.08	(0) 0.08	(−1) 30	302.87
2	8	(+1) 5	(0) 0.75	(0) 0.08	(0) 0.08	(+1) 40	270.48
2	9	(+1) 5	(0) 0.75	(0) 0.08	(+1) 0.10	(0) 35	302.87
2	10	(0) 4	(−1) 0.50	(0) 0.08	(−1) 0.05	(0) 35	412.80
2	11	(−1) 3	(0) 0.75	(0) 0.08	(+1) 0.10	(0) 35	259.50
2	12	(0) 4	(0) 0.75	(0) 0.08	(0) 0.08	(0) 35	368.40
2	13	(0) 4	(0) 0.75	(0) 0.08	(0) 0.08	(0) 35	394.20
2	14	(0) 4	(0) 0.75	(−1) 0.05	(0) 0.08	(−1) 30	78.85
2	15	(0) 4	(+1) 1.00	(−1) 0.05	(0) 0.08	(0) 35	297.80
2	16	(0) 4	(−1) 0.50	(0) 0.08	(+1) 0.10	(0) 35	154.50
2	17	(−1) 3	(0) 0.75	(0) 0.08	(0) 0.08	(−1) 30	145.50
2	18	(0) 4	(0) 0.75	(+1) 0.10	(0) 0.08	(−1) 30	190.47
2	19	(0) 4	(−1) 0.50	(+1) 0.10	(0) 0.08	(0) 35	336.00
2	20	(0) 4	(0) 0.75	(0) 0.08	(0) 0.08	(0) 35	377.90
2	21	(0) 4	(0) 0.75	(+1) 0.10	(0) 0.08	(+1) 40	382.30
2	22	(0) 4	(+1) 1.00	(+1) 0.10	(0) 0.08	(0) 35	323.60
2	23	(0) 4	(+1) 1.00	(0) 0.08	(−1) 0.05	(0) 35	405.70

[a] Lipase production is considered as the response.

Multiple regression analysis of the experimental data yielded the following second-order polynomial equation:

$$\begin{aligned}\text{Lipase activity, } Y\,(\text{U/ml}) &= 16.88 + 54.85X_1 + 34.74X_2 + 36.05X_3 - 74.21X_4 + 63.66X_5 \\ &\quad - 15.67{X_1}^2 - 5.03{X_2}^2 - 2.05{X_3}^2 + 9.94{X_4}^2 - 80.65{X_5}^2 \\ &\quad + 7.45X_1X_2 - 50.51X_1X_3 - 26.02X_1X_4 + 14.09X_1X_5 \\ &\quad - 5.80X_2X_3 + 30.93X_2X_4 - 17.70X_2X_5 - 31.22X_3X_4 \\ &\quad - 16.93X_3X_5 + 8.84X_4X_5\end{aligned}$$

The ANOVA analysis of lipase production showed a P value of 0.0057, which indicated the significance of the model. Three linear coefficients, incubation time (X_1), KH_2PO_4 (X_4) and temperature (X_5); and one quadratic term (${X_5}^2$) were significant model terms for the response.

The coefficient of determination (R^2) obtained by ANOVA was 0.8325, which indicated that a satisfactory adjustment of the quadratic model to the experimental data was ensured. Approximately 83% of the variability in the dependent variable (response) could be explained by the model. The coefficient of variance, indicative of the degree of precision with which the treatments are compared, had a value of 31.65%. The value of adjusted R^2 (0.6238) indicated a reliable agreement between the experimental and predicted values of lipase yield. All the selected parameters were significant and varied levels of interactions were recorded for the variables in their cumulative effect on lipase production. The adequate precision that measures the signal to noise ratio for the model was higher than 4 (6.065), which denoted a good fit. Thus, this model could be used to navigate the design space.

Analysis of factors influencing lipase production

Three-dimensional response surface curves were plotted to study the interaction among various physicochemical factors and to determine the optimum concentration of each individual factor for maximum lipase production by *A. awamori*. The model predicted maximum lipase production up to 482.85 U/ml that could be achieved using 0.8% (w/v) soyabean meal, 0.1 M $(NH_4)_2SO_4$ and 0.05 M KH_2PO_4 at 35 °C for five days of incubation. An overall 4.6-fold increase in lipase production was achieved after validation of RSM in shake flasks.

The pairwise interaction among the factors in terms of lipase production in the optimized set was assessed by examining the response surfaces. Three dimensional response surfaces were generated holding three factors constant at a time and plotting the response obtained for varying levels of the other two. The interaction effect of soyabean meal concentration and incubation temperature on lipase production (Fig. 4a) indicated that maximum lipase yield could be achieved at higher temperatures (36–38 °C) with 0.75% soyabean meal concentration. Data presented in Fig. 4b for the study on the interaction effect of incubation temperature and $(NH_4)_2SO_4$ concentration suggested that the maximal yields are supported by incubation temperatures 36–38 °C with 0.1 M of $(NH_4)_2SO_4$. In the case of interaction between incubation period and temperature on lipase production, a parabolic trend in response to variation in incubation temperature from 35 to 38 °C was noted recording a maximal activity at 37 °C and after five days of incubation (Fig. 4c). It was observed that maximum lipase production could be achieved at higher concentrations of soyabean and $(NH_4)_2SO_4$; and at central values of incubation temperature and KH_2PO_4 concentration.

From the response surface graphs, it is concluded that maximum production of lipase by *A. awamori* was supported by the optimum values of incubation temperature, soyabean meal, KH_2PO_4, $(NH_4)_2SO_4$ and incubation period which were in the range of 36–38 °C, 0.75–0.88%, 0.05 M, 0.1 M and five days, respectively.

Validation of the response surface model

Validation of the deduced response surface model based on the previous experiments was carried out in shake flasks under conditions predicted by the model. All the seven experimental sets studied indicated a close relationship between the predicted and experimental results. A maximum of 484 U/ml of lipase was obtained experimentally which was closer to the predicted value of 482.8 U/ml (data not shown). Hence, it is concluded that the model was successfully validated.

The optimized conditions for lipase production were as follows:

Soyabean meal-0.77% (w/v); $(NH_4)_2SO_4$-0.1 M; KH_2PO_4-0.05 M; rice bran oil-2% (v/v); $CaCl_2$-0.05 M; PEG 6000-0.05% (w/v); NaCl-1% (w/v); inoculum-1% (v/v); pH 3; incubation temperature-35 °C and incubation period-five days. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of the response surface methodology for process optimization. Among the different variables tested, incubation temperature was found to be the major significant factor influencing the production of lipase. It was also observed that lipase yield under optimized condition was nearly 4.6 times higher than that obtained under the unoptimized conditions. In the case of *Burkholderia cepacia* [41], an overall 2.5-fold increase in lipase production and a 1.8-fold increase in specific activity were achieved after validation of RSM in shake flasks. Similarly, after optimization using RSM up to a ninefold increase in lipase production for *Penicillium cyclopium* [42], 1.8-fold increase for *A. carneus* [14] and 3.14-fold increase for *Rhizopus delemar* [43] were reported.

Time course study under optimal conditions

Data obtained for the time course experiment conducted over a period of 192 hours (eight days) under optimized condition (Fig. 5) testify that the enzyme production commenced after 36 hours of incubation and reached a peak after 96 hours (495.0 U/ml). Further incubation beyond 108 hours did not favor enhanced enzyme activity and instead resulted in a decline. Maximal specific activity was recorded at 108 hours (1164.63 U/mg protein) and maximum biomass (29.5 mg/ml) was attained within 48 hours of incubation, which remained almost stable during the entire study period. Indeed maximal enzyme activity (96 hours) and maximal specific activity (108 hours) were recorded at two different incubation periods. It may be noted that the enzyme assay was done with crude culture filtrate which could contain other secretary proteins other than the extracellular lipase enzyme. Probably some of the secretary proteins present in the crude culture filtrate could have got auto-degraded and consequently the protein content declined at 108 hours and this decrease in protein content attributed to the slight enhancement in specific activity at 108 hours.

The use of RSM in the present study showed a decrease in the total time required for lipase production as 96 hours from the initial

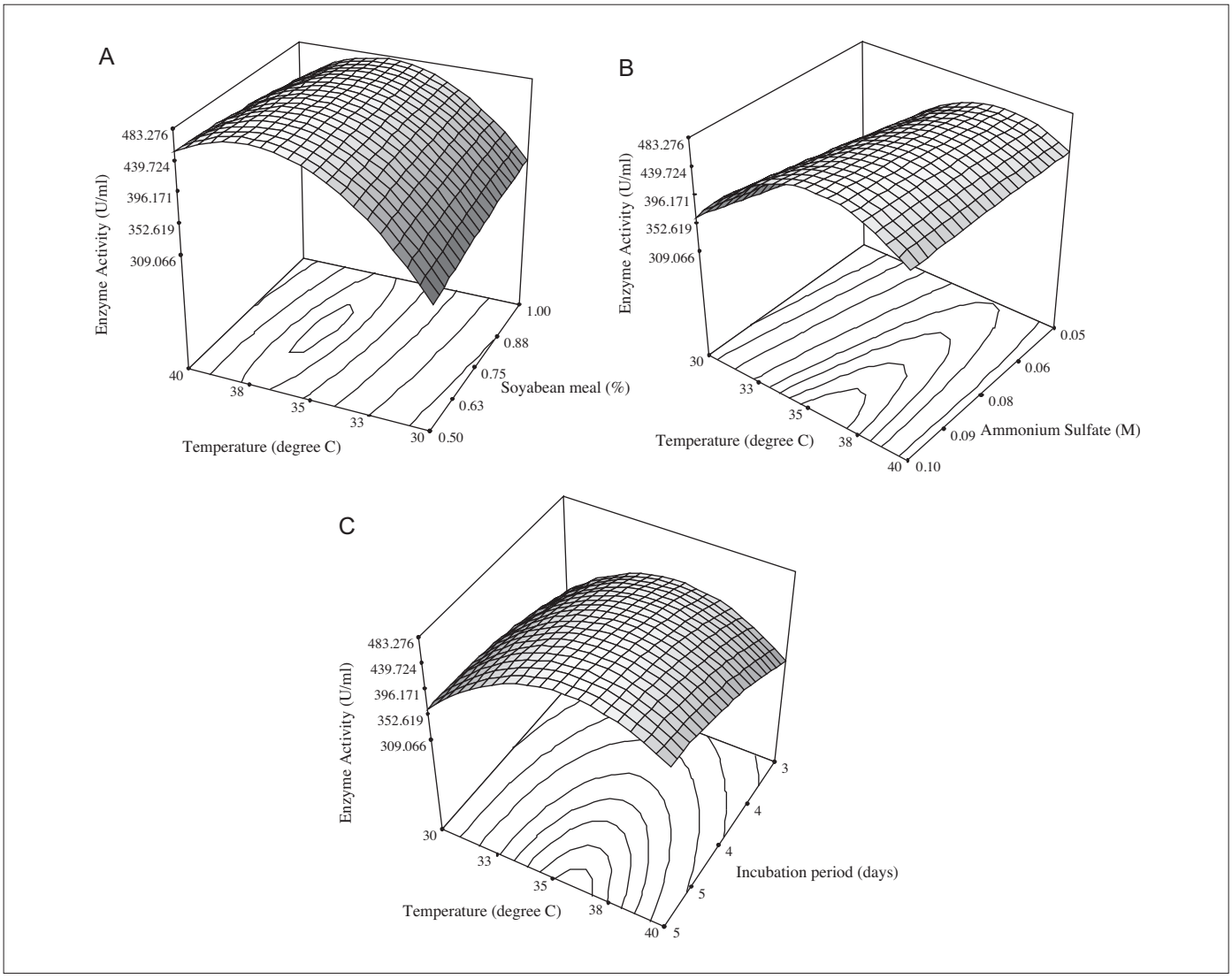

FIGURE 4

Response surface graphs depicting the interaction between **(a)** incubation temperature and soyabean meal concentration; **(b)** incubation temperature and $(NH_4)_2SO_4$ concentration and **(c)** incubation temperature and incubation period toward lipase production by marine *A. awamori* BTMFW032 based on Box–Behnken design.

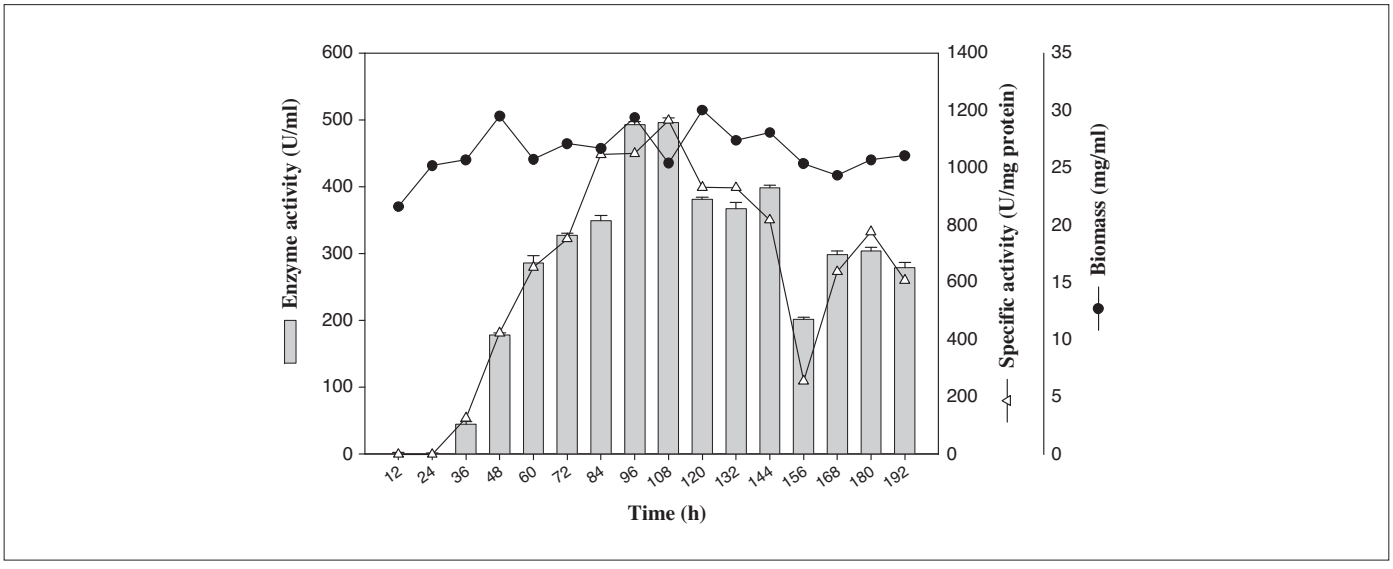

FIGURE 5

Time course study under optimized condition for lipase production by marine *A. awamori* BTMFW032.

TABLE 3

Yield and fold of purification of lipase produced by marine *A. awamori* BTMFW032

Sample	Total protein (mg/ml)	Total activity (U/ml)	Specific activity (U/mg)	Yield of protein (%)	Yield of activity (%)	Fold of purification
Crude extract	643.5	79,612	123.7	100	100	1
$(NH_4)_2SO_4$ fractionation 40–90%	21.24	32,712	1540.1	3.3	41	12.5
Ion Exchange chromatography (DEAE)	7.2	26,880	3733	1.1	33.7	30.2

120 hours obtained by the 'one factor per trial' approach (data not shown). Incubation periods up to 48 and 72 hours for lipase production have been reported for *Bacillus stearothermophilus* SB-1 [44] and *R. oligosporus* [45], respectively. Cihangir and Sarakaya [46] reported highest lipase activity on day 4 of incubation for *Aspergillus* sp., and similar results were also reported for *Penicillium roquefortii* [47] and *A. niger* [48]. In the present study protease assay was also conducted during the entire period of study and no protease was detected up to 192 hours of incubation. Hence, based on this observation it is inferred that the lipase produced by *A. awamori* is a 'protease free lipase' (Fig. 5), which conferred its high storage stability (data not shown). It was reported that proteases released into the medium, as a consequence of fermentation process, enhance lipase inactivation (9). In the present work, even though protease was not produced during the entire period of study, the observed decline in lipase activity after 108 hours may be due to pH inactivation by the fatty acid formed during lipase action on oil substrate. An alkaline, thermostable protease free lipase from *Pseudomonas* sp. [49] and lipase with similar properties from *A. carneus* [50] have been reported in the literature.

Enzyme purification and characterization

Results obtained for the purification of crude enzyme carried out in the present study (Table 3) indicated that 33.7% lipase (30.2-fold of purification) with a specific activity of 3733 U/mg protein could be recovered. The purified enzyme (obtained after ion exchange) showed a single polypeptide band on both native and SDS-PAGE analysis, with a molecular mass of approximately 90 kDa (Fig. 6a,b). Previous reports on the molecular mass of lipase were in the range between 11 and 67 kDa [51]. The zymographic analysis of the purified enzyme showed lipase activity as fluorescence under UV light, after staining with MUF butyrate. In contrast to other zymographic systems, the sensitivity range of MUF-butyrate technique is extremely high, allowing detection of 1.5×10^{-7} units of Pancrealipase® in less than 15 min. The short time required for activity detection on gels greatly contributes to prevent protein diffusion, thus allowing a most accurate determination of the protein molecular weight. An important advantage of this zymographic technique is that after activity detection, the same gels can subsequently be stained with a conventional dye to determine the molecular mass of the active proteins [24].

The zymogram showed three fluorescent bands in the case of $(NH_4)_2SO_4$ precipitated sample, indicating presence of more than one type of lipase in the fermented broth, compared to a single fluorescent band obtained with ion exchange purified sample (Fig. 6c). This observation raises a question what happened to the other two fractions which was not observed after ion exchange chromatography. Because the DEAE matrix was equilibrated with phosphate buffer pH 7.5, the fraction in the enzyme preparation which had optimum binding pH in this range could have got bound to the matrix and remained adsorbed to the matrix. Whereas, the other two lipase fractions whose optimal binding pH may be different might have got washed out as unbound proteins.

Results obtained for the purification steps confirmed the purity and activity of the purified lipase obtained from *A. awamori*. Most of the lipase purification schemes in the literature focused on purifying small amounts of the enzyme to homogeneity toward characterization of the same. Whereas, there is hardly any information published on large-scale processes for commercial purification of lipase. May be the reason is that most commercial applications of lipase do not require highly pure enzyme and excessive purification is expensive and reduces overall recovery of the enzyme [52].

Gel filtration chromatography of the $(NH_4)_2SO_4$ precipitated sample using Sephadex G100, yielded three peaks with lipase activity (Fig. 7). The molecular mass of the protein, calculated from the standard graph plotted for K_{av} versus log molecular weight of the standard proteins, indicated that the molecular masses of the enzyme for the three fractions (I, II and III) were approximately 90 kDa, 65 kDa and 38 kDa respectively. A similar observation was reported earlier in *Candida rugosa*, where three different peaks with different molecular masses were obtained by SDS-PAGE, which indicated the presence of different lipase isozymes [53]. It was also reported that more than 50% of lipase producing yeast produce the enzyme as various isozymes [54]. Similarly, Hama *et al.* [55] also reported production of two types of lipases by *Rhizopus oryzae*.

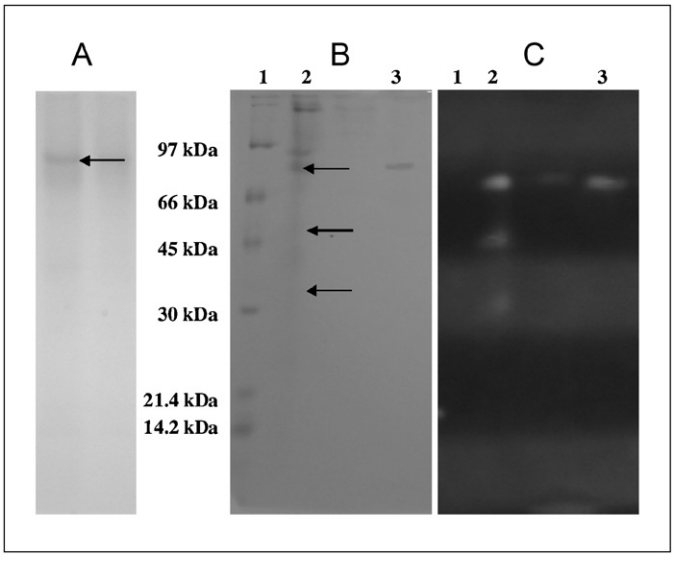

FIGURE 6

Electrophoretic analysis of lipase produced by marine *A. awamori* BTMFW032: **(a)** Native-PAGE, **(b)** SDS-PAGE and **(c)** Zymogram. Lane 1 – marker, Lane 2 – sample after $(NH_4)_2SO_4$ fractionation, and Lane 3 – sample after ion exchange chromatography.

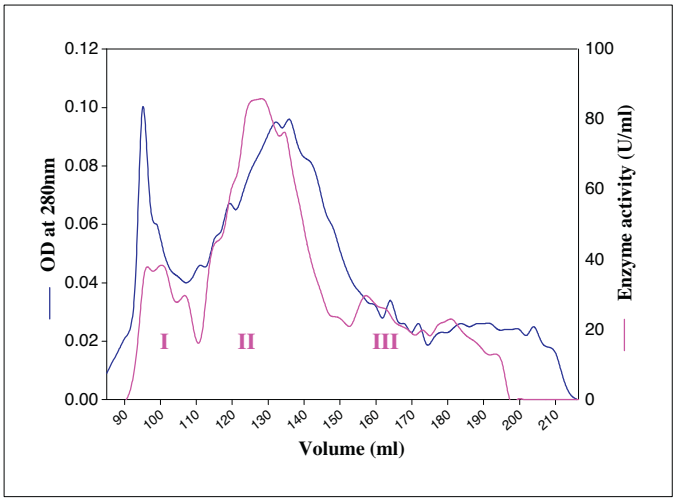

FIGURE 7

Elution profile of marine *A. awamori* BTMFW032 lipase on Sephadex G100.

Effect of pH and temperature on lipase activity

From the results obtained for the studies on effect of pH, it was inferred that optimum pH for this lipase enzyme was pH 7 at 40 °C. Further it was noted that, in general, the lipase was active over a pH range of 5–9 and an increase in pH from 5 to 7 led to proportionate increase in enzyme activity (data not shown). The enzyme was observed to become totally inactive at pH below 4 and above 10. Interestingly more than 60% of maximal enzyme activity was recorded in the pH range of 6–8, indicating slight alkaline nature for this lipase.

The enzyme was found to be active over a broad range of incubation temperatures although recording maximal activity at 40 °C (data not shown). Temperatures above 45 °C were observed to result in a sharp decline in enzyme activity, with a relative activity of 85% at 45 °C, 50% at 50 °C and 18% at 55 °C when compared to the optimal temperature (100% at 40 °C). In fact, the lipase activity was observed to show a linear increase along with increase in temperature, and the increase was rapid over a range of temperature varying from 20 °C to 40 °C, similar to lipases of *Fusarium oxysporum* [56], *Penicillium citrinum* [57], *Candida rugosa* [58], and *Fusarium solani* [59]. Nevertheless, the enzyme was found to have activity even at 5 °C with 31.85% relative activity. The data obtained for the study clearly indicated the preference of this enzyme for a higher temperature of 35–45 °C for maximal enzyme activity. Further it was also observed that the enzyme could record more than 50% of activity in the range of 20 to 50 °C. In general, lipases from *Aspergillus* strains were reported to be active between pHs 4 and 7 and at temperatures between 40 and 50 °C [60].

Oil laden effluent treatment with fungal lipase

Microbial lipases have drawn greater attention recently in industries and by environmental engineers and consultants, because of their wide range of applications; including their bioremedial potential for applications in environmental protection. Ayurvedic oil treatment is an ancient medical practice in India. To promote tourism ayurvedic massage parlors and hospitals are sprouting like mushrooms. A large amount of ayurvedic oil is discharged from these hospitals each day causing heavy pollution in fresh water and seawater. In this context, potential of lipase from marine *A. awamori* BTMFW032 was evaluated for the treatment of oil laden effluent as described under the section 'Materials and methods'. From the results obtained, it is inferred that there was 91.4% reduction in fat and oil content after treatment of ayurvedic oil, followed by palm oil (80.16%), fish fry oil (33.3%), coconut oil (30%) and dalda (22%). It was reported that the lipase of *Pseudomonas aeruginosa* LP602 added to lipid-rich restaurant wastewater reduced the lipid concentration to less than 10 mg/ml and the lipid fraction was degraded by 70% during the first 24 hours [61]. Based on the results obtained from our study, it is speculated that lipase from *A. awamori* BTMFW032 which showed potential for degrading ayurvedic oil has scope for probable application in oil laden effluent treatment and for the reuse of this wasted oil toward biodiesel production.

Conclusions

Marine microorganisms, with their unique nature differ very much in many aspects from their terrestrial counterparts and are known to produce diverse spectra of novel and useful substances. Among the different genera of fungi, *Aspergillus* is known as the dominating group for industrial production of enzymes, particularly lipase. It is concluded that *A. awamori* BTMFW032 isolated from seawater has potential for use in industries for the production of extracellular lipase under SmF, which could be used in bioremediation of oil laden effluent. Further the present study also endorses the belief that marine microorganisms are unique in terms of their characteristics and holds potentials for varied applications in industries and environmental protection.

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