3. RESULTS

3.1. ISOLATION AND IDENTIFICATION OF LIPASE PRODUCING BACTERIAL FLORA FROM THE INTESTINE OF MARINE FISH SARDIENELLA LONGICEPHS

3.1.1. Total viable count (TVC) and screening of lipase producing bacterial isolates

Substantial populations of aerobic bacterial strains were isolated from the fore and hindgut regions of the candidate fish S. longicephs. The results on the intestinal aerobic bacterial populations showed that the foregut has the TVC of $8 \times 10^5$ CFU/g and the hindgut has $3.1 \times 10^5$ CFU/g (Table 3.1). Totally 40 different morphologically dissimilar strains were isolated and the strength of extracellular lipase production by all the isolated bacterial strains was screened individually for their ability to produce lipase in sprit blue agar.

Among the 40 isolates, only 11 strains were found to be the producers of lipase in sprit blue agar. It was observed with clear zone around the colonies due to hydrolysis of tributyrin supplied in the medium (Plate 2.2). The total TVC of lipase producing communities in the foregut was $2 \times 10^4$ CFU/g and in the hindgut was $1.2 \times 10^3$ CFU/g (Table 3.1). The results showed that the intestinal tract of S. longicephs composed of considerable number of lipase producing bacterial communities.

3.1.2. Identification of lipase producing strains

Totally 11 extracellular lipase producing isolates were identified by using morphological, physiological and biochemical characteristics. Finally the identification was also made through 16S rRNA sequencing and analysis. The
results on the biochemical identification showed that most of the isolates (6 no’s) were belonging to the genera *Bacillus* and which resulted as Gram positive rods. Among the remaining 5 isolates, 3 isolates were Gram negative rod, and the other two isolates represent Gram positive cocci and Gram negative curved rod, respectively. Biochemical, morphological and physiological characteristics of these 11 isolates are presented in Tables 3.2 to 3.4. Conventional identification of the 6 *Bacilli* form bacteria showed different biochemical characteristics; also the other 3 Gram negative isolates identified were also having different biochemical features.

To confirm the identification process of the lipase positive 11 different isolates, 16S rRNA sequencing and analysis were made by using the forward primer 5’ AGAGTTTGATCCTGGCTCAG 3’ and reverse primer 5’ ACGGCTACCTTGTTACGACTT 3’. The result showed that all the isolates were different species. This was revealed from the blast search and construction of Neighbour-joining phylogenetic tree using MEGA 4.0 software. In accordance with the conventional method of identification, it was also confirmed that, majority of the isolates were *Bacilli* form through the molecular identification using 16S rRNA sequences. Figures 3.1 to 3.6 constructed by using MEGA 4.0 resulted that among the 11 isolates, 6 isolates belonging to the family Bacillaceae, which included the genera *Bacillus*, *Virgibacillus* and *Halobacillus*. Therefore, it occupied about 54% of total lipase producing strains isolated from the intestinal tract of *S. longicephs*. The other 3 Gram negative isolates were belonging to the family Halomonadacea, which included the genera *Halomonas* (Fig. 3.7) and *Cobetia* (Fig. 3.8 and 3.9). The last two isolates belonging to the family Vibrionaceae and Staphylococcaceae
respectively included the genera of *Vibrio* (Fig. 3.10) and *Staphylococcus* (Fig. 3.11).

The different forms of *Bacillus* isolates identified were *Bacillus subtilis* AP-MSU 6, *Bacillus cereus* AP-CMST, *Bacillus altitudinis* AP-MSU, *Virgibacillus* sp. AP-MSU 1, *Halobacillus trueperi* AP-MSU 9 and *Halobacillus* sp. AP-MSU 8 (Table 3.2 and Fig. 3.1 to 3.6). Similarly different species of isolates of Halomonadaceae family belonging to the genus *Halomonas* were named as *Halomonas* sp. AP-MSU 5, *Cobetia* sp. AP-MSU 3 and *Cobetia marina* AP-MSU 2 (Table 3.3 and Fig. 3.7 to 3.9). The remaining two isolates were named as *Vibrio furnisii* AP-MSU 7 and *Staphylococcus cohnii* AP-CMST (Table 3.4 & Fig. 3.10 and 3.11).

### 3.1.3. Occurrence and species composition of lipolytic bacteria from the intestine

Species composition of these 11 strains and their corresponding range in the gut region were calculated and presented in Table 3.5 and Fig.3.12. From this calculation, it was resulted that Bacillaceae family strains occupied 62% of the total number of lipolytic isolates. 31% of lipase producing communities belong to the family Halomonadaceae with the genus of *Halomonas* and *Cobetia*. Genus *Vibrio* and *Staphylococcus* composed of 4 and 3%, respectively. Out of 62% of Bacillaceae family, 15.8% and 15.7% of *Bacillus* strains were respectively belonging to the strain *Halobacillus* sp. and *Virgibacillus* sp. and these strains were observed in both foregut and hind gut regions of *S. logicephs*. *Bacillus subtilis* also constituted a major species (11%) among the 62% of *Bacillus* genera and it was also isolated from both fore and hind gut regions. Considerable percentage (10%) of
*Halobacillus trueperi* was isolated from the hind gut region and its occurrence in the foregut region was absent. *Bacillus altitudinis* and *Bacillus cereus* were also contributed 4% and 5.5%, respectively among the total isolates of genus *Bacillus*. *Bacillus altitudinis* was isolated from both the gut regions, but *Bacillus cereus* was observed only in the foregut region.

Next to *Bacillus*, the family Halomonadaceae including the genera *Halomonas* and *Cobetia* occupied 31% of total lipolytic strains. Among these two genera, the strain *Halomonas* sp only observed in the foregut region and composed of 12.3% of the total Halomonadaceae family strains isolated. Next to this *Cobetia* sp (7.5%) and *Cobetia marina* (11.2%), totally composed of 18.7% of Halomonadaceae family strains. Among these two strains, *Cobetia* sp observed in both the gut regions, but *Cobetia marina* was observed only in hindgut region of the fish.

The other two strains such as *Vibrio furnisii* and *Staphylococcus cohnii* were only isolated from the fore gut region, which composed of least percentage of occurrence when compared to the families Bacillaceae and Halomonadaceae. Also these two strains were respectively composed of 4 and 3% of total lipase producing isolates.

### 3.1.4. Effect of different organic solvents on lipase production by the selected isolates

The capacity of utilization of organic solvents for lipase production by the selected isolates was studied by using seven organic solvents such as DMSO, acetone, hexane, chloroform, benzene, ethanol and methanol at 5% level and the
medium devoid of the above said solvents was maintained as control for each species.

3.1.4.1. *Bacillus subtilis* AP-MSU 6

The result on the effect of organic solvents on lipase production by *B. subtilis* AP-MSU 6 is presented in Fig. 3.13. Among the tested organic solvents, the lipase production ability by *B. subtilis* was more only in acetone (123.06 U/ml) supplied medium, when compared to that of control (112.0 U/ml). In other solvents, the lipase production was considerably reduced, however in DMSO and hexane supplemented media, the lipase production completely inhibited. When compared with control, other solvents like benzene (79.25 U/ml) and ethanol (45.99 U/ml) retained 70% and 43% of lipase production, respectively. But others considerably reduced the lipase production. The statistical one-way ANOVA revealed that the lipase production as a function of variation between organic solvents was highly significant (F = 5748.81: P < 0.0001) (Table 3.6).

3.1.4.2. *Bacillus cereus* AP-CMST

Effect of different organic solvents on lipase production by the isolate *B. cereus* AP-CMST is presented in Fig. 3.14. The result showed that this species could utilize majority of supplemented solvents for lipase production. The lipase production by this species was high (113.16 U/ml) in ethanol supplied medium and it was about two fold higher than the lipase production observed in control (63.2 U/ml) medium. Among the other supplemented solvents, it can tolerate and produced maximum lipase in acetone (88.08 U/ml), hexane (83.76 U/ml) and DMSO (63.41 U/ml), when compared with control. But the media supplemented
with chloroform (23.54 U/ml), benzene (48.91 U/ml) and methanol (32.19 U/ml) reflected less lipase production than control. The one-way ANOVA test conducted on lipase production by *B. cereus* as a function of variation due to organic solvents was statistically more significant (*F* = 1903.65; *P* < 0.0001) (Table 3.6).

### 3.1.4.3. *Bacillus altitudinis* AP-MSU

The result on the effect of various solvents on lipase production by *B. altitudinis* AP-MSU showed that, this isolate having good tendency to utilize most of the supplied solvents such as hexane (68.9 U/ml), acetone (60.85 U/ml), chloroform (58.57 U/ml) for maximum production of lipase over the control (50.9 U/ml), the percentage increase of lipase production in the above three solvents supplied media was respectively 35%, 19% and 15% than the control. Among the other solvents, the lipase production was highly reduced in benzene (17.1 U/ml) supplied medium. Similarly in DMSO (38.78 U/ml) and methanol (39.54 U/ml) supplemented media, the lipase production was retained to 76% and 77%, respectively over the control (Fig. 3.15). The statistical one-way ANOVA revealed that the lipase production by *B. altitudinis* as a function of variation between organic solvents was highly significant (*F* = 9790.441; *P* < 0.0001) (Table 3.6)

### 3.1.4.4. *Virgibacillus* sp. AP-MSU 1

The effect of different solvents on lipase production by *Virgibacillus* sp. AP-MSU 1 resulted that, except DMSO (155.95 U/ml), all the other solvents found to be decreased the lipase production than the control (152.3 U/ml). Among the solvents screened, hexane (3.9 U/ml) was highly inhibited and about 98% of lipase production was reduced than control. The inhibition by ethanol (109.4 U/ml) was
slightly low and about 72% of lipase production was retained when compared with control. In other solvents, lipase production was significantly reduced (Fig. 3.16). The one-way ANOVA resulted that the lipase production by *Virgibacillus* sp. as a function of variation between organic solvents was statistically more significant (F = 3338.562; P<0.0001) (Table 3.6).

### 3.1.4.5. *Halobacillus trueperi* AP-MSU 9

The effect of organic solvents on lipase production by *H. trueperi* AP-MSU 9 resulted that all the solvents except ethanol (49.47 U/ml) and DMSO (34.14 U/ml), drastically inhibited the lipase production when compared to that of control (35.67 U/ml) (Fig. 3.17). Lipase production by this strain was completely inhibited in acetone and methanol supplemented media. Very less amount of lipase production was observed in hexane (10 U/ml), chloroform (7 U/ml) and benzene (12 U/ml) supplied media. The statistical one-way ANOVA conducted on lipase production by *H. trueperi* as a function of variation between organic solvents was highly significant (F = 1198.234; P<0.0001) (Table 3.6)

### 3.1.4.6. *Halobacillus* sp. AP-MSU 8

The lipase production by *Halobacillus* sp. AP-MSU 8 in all the organic solvents supplied media considerably increased than the control (55.1 U/ml). This strain tolerated and utilized all the organic solvents for increasing the lipase production. Solvents like chloroform (141.65 U/ml), ethanol (141.61 U/ml) and hexane (122.3 U/ml) were highly utilized by this strain and 2-3 fold lipase production was increased when compared with control (Fig.3.18). The statistical one-way ANOVA conducted on lipase production by *Halobacillus* sp. as a function
of variation between organic solvents was highly significant (F = 8299.096; P<0.0001) (Table 3.6)

**3.1.4.7. Halomonas sp. AP-MSU 5**

The result on the effect of different organic solvents on lipase production by *Halomonas* sp. AP-MSU 5 is represented in Fig. 3.19. It showed that this strain could not tolerate in all the supplied organic solvents, and in all the organic solvents supplied media, lipase production was considerably decreased over the control (111.74 U/ml). Among the tested solvents, lipase production was extremely low (7.9 U/ml) in benzene supplemented medium than others. More than 70% of lipase production was reduced in organic solvents added media when compared to that of control. The one-way ANOVA conducted on lipase production by *Halomonas* sp. as a function of variation between organic solvents was statistically more significant (F = 1929.079; P<0.0001) (Table 3.6).

**3.1.4.8. Cobetia sp. AP-MSU 3**

The influence of different organic solvents on lipase production by *Cobetia* sp. AP-MSU 3 is given in Fig. 3.20. It was understood that this strain could able to produce good amount of lipase by utilizing majority of organic solvents. Most of the solvents such as chloroform (85.015 U/ml), methanol (66.34 U/ml), benzene (75.8 U/ml) and acetone (65.22 U/ml) were found to be either increased or retained the lipase production when compared with control (67.94 U/ml). Only two solvents like DMSO (11.5 U/ml) and ethanol (12.68 U/ml) were found to be not favored on lipase production. In hexane supplied medium also the lipase production was decreased, however it retained 67% of lipase when compared to that of control. The
The statistical one-way ANOVA conducted on lipase production by *Cobetia* sp. as a function of variation between organic solvents was highly significant (F = 7284.063; P<0.0001) (Table 3.6)

3.1.4.9. *Cobetia marina* AP-MSU 2

The result on the effect of various organic solvents on lipase production by *C. marina* AP-MSU 2 showed that this strain could utilize only one organic solvent namely acetone (146.96 U/ml) and the other solvents were found to be decreased the lipase production than the control (130.865 U/ml). But in hexane supplemented medium, the lipase production was completely inhibited (Fig. 3.21). The inhibition of lipase production was moderate in chloroform (120.34 U/ml) and methanol (109.75 U/ml) supplied media, however over 85% of lipase production was retained. Other solvents like benzene (54.91 U/ml), DMSO (51.77 U/ml) and ethanol (64.8 U/ml) recorded less than 50% of lipase production than control. The one-way ANOVA conducted on lipase production by *C. marina* as a function of variation between organic solvents was statistically more significant (F =18512.09; P<0.0001) (Table 3.6)

3.1.4.10. *Vibrio furnisii* AP-MSU 7

Figure 3.22 represents the effect of organic solvents on lipase production by the isolate *V. furnisii* AP-MSU 7. The result showed that this strain was highly sensitive to organic solvents. No lipase production was observed in most of the solvents including DMSO, hexane, benzene, ethanol and methanol. This strain could able to tolerate only in acetone supplemented medium and produced moderate level of lipase (38.94 U/ml) than control (24.53 U/ml). In chloroform
supplemented medium, very less amount of lipase production was observed (4.94 U/ml). The one-way ANOVA conducted on lipase production by *V. furnisii* as a function of variation between organic solvents was statistically more significant (F =1088.813; P<0.0001) (Table 3.6)

### 3.1.4.11. *Staphylococcus cohnii* AP-CMST

The result on the influence of the supplementation of organic solvents on lipase production by the isolate *S. cohnii* AP-CSMT is showed that acetone (188.51 U/ml) and methanol (153.30 U/ml) were highly utilized by this strain and the lipase produced by this strain was maximum over the control (114.84 U/ml) (Fig. 3.23). Whereas the other solvents found to decrease the lipase production and inhibition of lipase production was observed more in chloroform (60.9 U/ml) and benzene (84.87 U/ml) supplied media. In hexane (102.43 U/ml), DMSO (103.83 U/ml) and ethanol (100.76 U/ml) supplied media, more than 90% of lipase production retained. The one-way ANOVA conducted on lipase production by *S. cohnii* as a function of variation between organic solvents was statistically more significant (F =1703.484; P<0.0001) (Table 3.6)

### 3.2. PRODUCTION, PURIFICATION, CHARACTERIZATION AND APPLICATION OF LIPASE PRODUCED BY *HALOBACILLUS* SP.

In the preliminary screening study, among the tested 11 lipase positive strains, *Halobacillus* sp APMSU 8 was tolerated almost in all the organic solvents and comparatively produced more amount of lipase than the other strains, therefore this particular strain alone was further characterized for lipase production and purification.
3.2.1. Preliminary Screening of nutritional factors

3.2.1.1. Effect of different supplementary carbon sources on lipase production

Carbon source is one among the important fermentation nutrients; it significantly alters the lipase production. The ability of lipase production with various supplementary carbon sources revealed that, majority of tested carbon sources was unhelpful for lipase production over the control (71.115 U/ml) (Fig. 3.24). Except dextrose (76.08 U/ml) and xylose (72.03 U/ml), all other tested carbon sources were produced negative impact on lipase production and also lipase production was completely inhibited in sucrose supplementation. In the case of dextrose and xylose, a margin of production was increased over the control and margin of increase in lipase production estimated over control was 7 and 2%, respectively for dextrose and xylose.

3.2.1.2. Effect of different supplementary nitrogen sources on lipase production

The result on the effect of various supplementary nitrogen sources on lipase production by *Halobacillus* sp is given in Fig.3.25. The result showed that three organic nitrogen sources namely casein enzyme hydrolysate (tryptone), yeast extract and beef extract induced better lipase synthesis over the control (66.6 U/ml). Whereas, the other supplied nitrogen sources reduced the lipase production than the control. Among the three best nitrogen sources, casein enzyme hydrolysate played a prominent role in lipase production (111.19 U/ml), followed by beef extract (88.09 U/ml) and yeast extract (81.12 U/ml). On the other hand, the lowest lipase production was observed in two inorganic nitrogen sources supplied media namely sodium nitrate (14.28 U/ml) and potassium nitrate (15.46 U/ml) and the lipase
production was only 12 and 13%, respectively when compared with the highest value observed in casein enzyme hydrolysate.

3.2.1.3. Effect of different supplementary triglycerides on lipase production

The effect of various triglycerides on solid state fermentation of lipase production was tested (Fig. 3.26). It revealed that most of the triglycerides have profound effect on lipase synthesis by this bacterium over the control (60.53 U/ml). Among the triglycerides tested, lipase production was recorded maximum in coconut oil supplied medium (116.43 U/ml), however the lowest production was observed in cod liver oil supplied medium (53.53 U/ml), and it was 55% lower when compared with highest production observed in coconut oil. Next to coconut oil, neem oil (101.28 U/ml) displayed a better supplementation of lipase production by this strain.

3.2.1.4. Effect of different surfactants on lipase production

The effect of various surfactants on lipase production was studied and the result is displayed in Fig. 3.27. It indicated that only poly ethylene glycol (79.16 U/ml) produced positive effect on lipase production over the control (72.41 U/ml). The other surfactants were considerably reduced the lipase production over the control. Very poor lipase production was observed in SDS (5.92 U/ml) supplied medium, and in this medium about 94% lipase production inhibited when compared to control. The other surfactants such as Triton X 100 (32.01 U/ml), Tween 20 (24.78 U/ml) and Tween 80 (28.33 U/ml), supplied media were also displayed lowest amount of lipase production than the control.
3.2.1.5. Effect of different metal ions on lipase production

The effect of various metal ions on lipase production was studied and the result is provided in Fig. 3.28. The result indicated that maximum amount of lipase production was found in magnesium chloride supplied medium (79.91 U/ml), followed by magnesium sulphate (52.21 U/ml) and calcium chloride (31.02 U/ml) were influenced positively on lipase production over the control (23.72 U/ml). The other metal ions were considerably reduced the lipase production, in particular, lipase production was completely arrested in ferric chloride supplied medium.

3.2.2. Effect of different physiological factors on lipase production

3.2.2.1. Effect of sodium chloride on lipase production

Sodium chloride is an important nutrient factor and it played an osmotic balance, because the bacterium was isolated from the gut of marine fish. The effect of sodium chloride on lipase production was determined and the result is given in Fig. 3.29. It indicated that 1.5 M salt concentration showed optimum for maximum (94.68 U/ml) lipase production by this bacterium. This concentration was estimated about 9% NaCl and above this level, the lipase production was gradually reduced. The production was relatively very low at 2.5 M (46.72 U/ml) and 3.0 M (20.12 U/ml) NaCl concentrations and it was about 49 and 21% lipase production, respectively when compared to that of 1.5 M sodium chloride.

3.2.2.2. Effect of different initial medium pH on lipase production

The result on the effect of initial medium pH on lipase production is reprecented in Fig.3.30. In the present study, the initial pH of the medium played a vital role in the lipase production and it was optimum at the pH range of 8-9,
However, it was specifically high at pH 9 (90.43 U/ml). Here high acidic or alkaline pH did not support the fermentative production of lipase. At high acidic (pH 3) or high alkaline (pH 12) pH, the lipase production was found to be very low (18.73 and 9.0 U/ml, respectively). This was estimated about only 20 and 10% of the lipase production observed than the optimum pH of 9.0.

3.2.2.3. Effect of different incubation temperature on lipase production

The effect of incubation temperature on lipase production was done by testing at various temperatures (20, 30, 40, 50, 60, 70 and 80°C). The result showed that the production was maximum at 40°C (66.9 U/ml) and above this temperature, the lipase production reduced gradually. In this study the temperature range between 30 and 40°C favored much for optimum lipase production and at 50°C (42.6 U/ml), the lipase production retains only 63% than observed at 40°C. Also at the tested higher temperature of 80°C, the lipase production drastically decreased (8.9 U/ml) and only 13% production was observed when compared to optimum temperature of 40°C (Fig.3.31).

3.2.3. Statistical optimization of nutritional factors on lipase production

3.2.3.1. Screening of nutritional factors using Placket-Burman Design

In the present study Plackett - Burman Design was used to identify the critical medium components having significant effect on lipase production by Halobacillus sp. The experiments were conducted by using 10 factors for 12 trials for lipase production. The statistical analysis with STATGRAPHICS Centurion XV statistical package clearly showed the demarked variation from 24.9 to 174.62 U/ml.
of lipase production corresponding to the variation in medium composition (Table 3.7).

The main effects of examined factors on lipase production were calculated and are presented in Table 3.8. The maximum influencing factors for lipase production were selected by using their P-values. The result showed that neem oil, yeast extract, casein enzyme hydrolysate (trypotone), coconut oil and poly ethylene glycol were produced positive effect on lipase production, which are statistically significant (P<0.05). Among the nutrients screened, dextrose, xylose, potassium dihydrogen ortho phosphate, magnesium chloride and beef extract produced very lower effect on lipase production, which are statistically non significant (P>0.05).

In this study, variables with confidence levels greater than 95% were considered as significant. Among the positive nutrients, neem oil (P=0.0163), yeast extract (P=0.0260), trypotone (P=0.0278), coconut oil (P=0.0323) and poly ethylene glycol (P=0.0336) proved to have the most profound influence on lipase production with significant P-value and they occupied first five places respectively. Table 3.9 shows the Analysis of Variance (ANOVA) for linear model on effect of independent variables on lipase production using factorial design. The effect of significant and most important variables on lipase production is given by the model in equation:

\[
\text{Lipase} = 113.714 + 63.4967*\text{Yeast extract} - 39.4733*\text{Casein enzyme hydrolysate} + 11.4792*\text{Beef extract} + 17.33*\text{dextrose} + 53.5917*\text{xylose} + 63.8375*\text{Coconut oil} - 253.158*\text{neem oil} - 163.189*\text{PEG} - 47.15*\text{MgCl}_2 - 153.125*\text{KH}_2\text{PO}_4
\]
The P-value in the ANOVA table is 0.0407; there is a statistically significant relationship between the variables at 95.0% confidence level.

The R-squared statistics indicated that the model as fitted explains 99.9719% of the variability in lipase. The adjusted R-squared statistics, which was more suitable for comparing models with different numbers of independent variables, was 99.6911%. The standard error of the estimate showed the standard deviation of the residuals to be 2.24301. The mean absolute error (MAE) of 0.6475 was the average value of the residuals. The effect of medium components on lipase production was also clearly understood by using Pareto chart (Fig. 3.32). It also indicated that the significant influence of neem oil, yeast extract, tryptone, coconut oil and polyethylene glycol on lipase production.

3.2.3.2. Optimization of lipase production by using Box–Behnken design

From the preliminary screening experiments using Plackett-Burman (PB) design, the maximum influencing factors such as neem oil, yeast extract and casein enzyme hydrolysate (tryptone) were selected for further optimization with Box–Behnken design. For each run, the experimental responses along with the predicted response obtained from the regression equation for the 15 combinations of three nutrient factors are shown in Table 3.10. This resulted that the observed values of lipase were close to predicted values. The results were analyzed by standard analysis of variance. The output showed the result of fitting a multiple linear regression model to describe the relationship between lipase and three independent variables. The equation of the fitted model is:
\[
\text{Lipase} = 122.264 - 143.053A + 48.1419B + 154.306B + 82.0558AA - \
49.7033AB + 15.532AC + 19.6959BB - 14.28BC - 77.8651CC
\]

In which A, B and C were respectively for neem oil, yeast extract and casein enzyme hydrolysate. The results were analyzed using the analysis of variance (ANOVA), and the results are summarized in Table 3.11. The model F-value of 3.64 and P-value of 0.0482 for lipase production showed that the model is significant and showed good fit of the models with the experimental data. The quality of fit of the model was checked by coefficient of determination ($R^2$). The R-squared statistics indicated that the model as fitted explains 97.6192% of the variability in lipase. The adjusted R-squared statistics, which was more suitable for comparing models with different numbers of independent variables, was 93.3337%. The standard error of the estimate showed the standard deviation of the residuals to be 8.66445. The mean absolute error (MAE) of 3.81011 was the average value of the residuals.

The P-value was used to check the significance of each of the coefficients. The responses taken from Table 3.12 revealed that linear coefficients: neem oil (P=0.0065), yeast extract (P=0.0003) and tryptone (P=0.0238), and quadratic coefficient: AA (P=0.0061), AB (P=0.0077) and CC (P=0.0011) have remarkable effects on the lipase production. Moreover among the linear coefficient, yeast extract (B) was more significant than the other factors. The result showed that concentrations of all the three nutrients have direct relationship with lipase production. This is represented in Pareto chart (Fig. 3.33).
The response surface plot would be of more helpful in understanding both the main and the interaction effects of maximum influencing factors. In order to determine the optimum levels of each variable for maximum lipase production, three-dimensional response surface plots were constructed by plotting the response (lipase production) on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels (zero coded level). The yield values for different concentrations of the variables can also be predicted from the respective response surface plots. The maximum predicted yield is indicated by the surface restricted in the response surface diagram.

Based on the regression equation, the response plot is shown in Fig. 3.34. Here neem oil and yeast extract concentrations were considered as independent variables while the tryptone was kept at zero coded level. The interaction between yeast extract and neem oil was observable and the lipase production increased with increase in yeast extract concentrations and lipase production was optimum with initial concentration of neem oil. Fig. 3.35 represents the effect of concentrations of neem oil and tryptone, while the yeast extract was maintained at zero coded level. Similar to previous plot, increase in lipase production was observed with increased in concentrations of tryptone and lipase production was also observed optimum with initial concentration of neem oil. A different observation was made in yeast extract concentration with tryptone concentrations (Fig. 3.36). In this plot increase in lipase production was observed with increase in concentration of yeast extract and tryptone. From the response plot obtained from Box–Behnken design, the optimum concentration of neem oil, yeast extract and tryptone resulted were 0.5,
2.0 and 0.85%, respectively. With these optimum concentration levels, the maximum lipase production observed was 253.84 U/ml.

3.2.4. Purification of lipase

The purification step was begun with ammonium sulphate precipitation of crude culture filtrate to achieve 80% saturation. In this step 76% enzyme yielded and 1.13% fold purity was obtained. Then it was subjected to DEAE-Sepharose Fast Flow anion exchange chromatography. The target lipase was eluted at the concentration between 0.4 M and 0.6 M NaCl and the elution profiles are represented in Fig. 3.37. In this step 47% enzyme yielded and 3.01 fold purification was obtained. Then the active samples from anion were further subjected to gel filtration with Sephadex G-75 and the elution was monitored (Fig. 3.38). In this final step 25% enzyme yielded and 10.19 fold purity was obtained (Table 3.13). The purity of lipase was checked by using SDS-PAGE with standard markers as mentioned earlier and the molecular mass of purified lipase recorded was 25 kDa (Fig. 3.39).

3.2.5. Characterization of purified lipase

3.2.5.1. Effect of pH on lipase activity

Figure 3.40 represents the effect of pH on the activity of purified lipase, and it resulted that it was optimum at pH 9.0. Since the activity was more at pH 9.0, therefore the nature of the enzyme was confirmed as alkaline lipase. The lipase started to act above pH 7.0 and in this pH about 50% of activity was found, then it started to increase up to pH 9.0. Finally it sharply decreased at pH 10.0 and here it retained only 77% activity.
3.2.5.2. Effect of temperature on lipase activity

Effect of temperature on lipase activity was studied by using different temperature range (10-80°C) and the optimum range of lipase activity was found at 30-40°C, also it was particularly high at 40°C (Fig. 3.41). The activity sharply decreased above this range, but at the same time 78% activity retained at 50°C. This lipase unable to tolerate at higher temperatures and only 20% and 8% activity was retained in 70 and 80 °C, respectively.

3.2.5.3. Effect of NaCl on lipase activity

NaCl is one of the important activator for enzymes from marine origin and the effect of NaCl on purified lipase activity was studied (Fig. 3.42). From the result, it was understood that 2-2.5 M NaCl was optimum range for maximum lipase activity and the activity particularly high at 2.5 M. Since, the activity was high at 2.5 M NaCl, therefore it was considered as highly halophilic lipase. The activity of lipase was reduced when the NaCl molar concentrations increased and it retained only 40% activity at 3.5 M concentration of NaCl.

3.2.5.4. Effect of metal ions on lipase activity

Metals like magnesium sulphate and barium chloride were found to increase the lipase activity by *Halobacillus* sp. Apart from these, the other metals found to decrease the enzyme activity (Table 3.14). Also magnesium chloride retains almost 90% activity from control. But in HgCl₂ and CuSO₄ substituted media, moderate inhibitory activity was found, whereas complete inhibition of lipase activity was observed in both ZnSO₄ and ZnCl₂ substituted media.
3.2.5.5. Effect of different surfactants on lipase activity

The effect of surfactants on the activity of purified lipase was studied by using seven different surfactants; it was observed that only two surfactants such as PEG and Tween 20 were influenced to increase the activity of purified lipase. But other tested surfactants were found to inhibit the lipase activity (Fig. 3.43). Among these, high inhibition (76%) was found in SDS supplied medium, when compared to control. In the case of other tested surfactants, 40-50% of inhibition activity recorded over control.

3.2.5.6. Effect of different organic solvents on lipase activity

The effect of different organic solvents on lipase activity was studied with seven different solvents at two different concentrations (10% and 20%). The result showed that this lipase can tolerate and work well with acetone and benzene at 10% level and with these two solvents, the lipase activity increased to 123 and 185%, respectively. The other solvents could not improve the enzyme activity; however they reduced the enzyme activity. Though in hexane, 84% lipase activity was retained, but others found to inhibit drastically at 10% level. At 20% level, all the solvents inhibited the enzyme activity, and drastic inhibition (41%) obtained in acetone, which was best at 10% level. But this enzyme worked better with some solvents when compared with their 10% level. Lipase activity slightly increased with ethanol, DMSO and chloroform, when compared to their initial concentration of 10% level (Table 3.15).
3.2.6. Application of lipase in PUFA concentrates production from trash fish (S. capistratus) liver oil

3.2.6.1. Degree of hydrolysis of S. capistratus liver oil

Before hydrolysis, the saponification and acid values of unhydrolysed S. capistratus liver oil resulted that 180.1 mg/KOH and 3.5 mg/KOH, respectively. The hydrolysis of trash fish oil with the lipase enzyme of Halobacillus sp. increased the acid value gradually. At the beginning (1 h) of hydrolysis, the acid value determined was 41.5 mg/KOH and further it increased gradually up to 8 h of hydrolysis (106.8 mg/KOH). After 8 h of hydrolysis, the acid value reached a stable condition, and further it was not increased much (Table 3.16). As the acid value increased, the corresponding hydrolysis percentage was also increased gradually from 1 to 8 h and then become stable. At the beginning of hydrolysis (1h), the percentage hydrolysis of oil was 21.51%, further the incubation period prolonged; the percentage hydrolysis was also increased gradually. For instance, at 2, 6 and 8 h of incubation, the percentage hydrolysis was increased to 42.8, 47.11 and 54.53%, respectively. Beyond the 8 h of incubation, the percentage hydrolysis recorded stable, i.e., during 12th h of incubation only 61.09% of hydrolysis obtained (Fig. 3.44).

3.2.6.2. Comparison of fatty acid composition of hydrolyzed and unhydrolyzed S. capistratus liver oil

The result on fatty acid composition of hydrolysed and unhydrolyzed S. capistratus liver oil is presented in Table 3.17. It showed that unhydrolyzed oil has more amount of saturated fatty acids (SFA) (41.34%). Myristic acid (C14:0) (13.54%) and palmitic acid (C16:0) (15.29%) were the major fatty acids observed...
in SFA group. Apart from these two SFA, stearic acid (C18:0) observed with high (8.27%) level. The mono unsaturated fatty acids (MUFA) level of unhydrolysed oil was 38.96%. Among these, the major MUFA observed were palmitoleic acid (C16:1) (20.18 %) and oleic acid (C18:1n9) (13.46%). The poly unsaturated fatty acids (PUFA) constituted only19.7%, and among these, the composition of eicosapentaenoic acid (C20:5n3; EPA) and docosahexaenoic acid (C22:6n3; DHA) were 6.78% and 2.42%, respectively. The other PUFA observed were linoleic acid (C18:2n6) (5.02%), α-linolenic acid (C18:3n3) (3.33 %) and arachidonic acid (C20:4n6) (2.15%).

After hydrolysis of oil with lipase, the total PUFA content of the oil was increased considerably. It was increased from 19.7% to 33.22%. The percentage of EPA was increased from 6.78 to 14.4% and the percentage of DHA increased from 2.42 to 4.6%. Also the percentage of linoleic acid (5.02 to 6.10%), α-linolenic acid (3.33 to 4.88 %) and arachidonic acid (2.15 to 3.24 %) were increased considerably. At the same time, percentage of SFA was decreased from 41.34 to 30.38% and also the percentage of MUFA decreased from 38.96 to 36.4%. This showed that the SFA and MUFA would have been converted to PUFA especially EPA and DHA. In SFA major percentage reduction observed was in myristic acid, heptadecanoic acid, palmitic acid and stearic acid. The capric acid and pentadecanoic acid were not detected after hydrolysis. In MUFA, the major percentage reduction was observed in erucic acid and oleic acid, when compared to unhydrolyzed oil.