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Original Article

OPTIMIZATION AND PURIFICATION OF LIPASE THROUGH SOLID STATE FERMENTATION BY BACILLUS *CEREUS* MSU AS ISOLATED FROM THE GUT OF A MARINE FISH *SARDINELLA LONGICEPS*

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ABSTRACT

A lipase producing bacterium, *Bacillus cereus* MSU AS was isolated from the gut of a marine fish *Sardinella longiceps*. In total, seven agricultural waste substrates (coconut oil cake, neem oil cake, onion skin waste, wheat bran, jack fruit waste, banana skin peels and pomegranate skin peel) were selected for solid state production of lipase by *B. cereus* MSU AS. Among the tested substrates, onion skin waste supplemented medium was found to be yielded maximum lipase and hence it was selected as a major substrate for further study. On optimization, 10% moisture content, 0.5% maltose (carbon source), 0.5% ammonium hydrogen carbonate (nitrogen source), 0.4% gingilly oil (triglyceride) and 9% NaCl concentration were favoured more on production of lipase by *B. cereus* MSU AS. Further the lipase produced by the candidate strain was purified and its molecular weight was determined as 49 kDa by SDS-PAGE analysis.

Keywords: *S. longiceps*, Gut bacterium, Lipase optimization, *B. cereus*, Solid state fermentation.

INTRODUCTION

Lipases are enzymes belonging to the group of the hydrolases, whose main biological function is to catalyze the hydrolysis of insoluble triacylglycerols to free fatty acids, mono and diacylglycerols and glycerol. Microbial lipases are biocatalysts that have interesting characteristics, as action under mild conditions, stability in organic solvents high substrate specificity and rigor enantioselectivity [1]. From the industrial point of view, lipases are considered much important, due to their greater production potential on a large scale [2]. Lipases have been proven efficient and selective biocatalyst in many relevant industrial applications like peptide synthesis, biosurfactant production and resolution of racemic mixtures or addition in detergents to produce optically active compounds [3]. Moreover, lipases of microbial origin are the most versatile enzymes which are commercially significant [4]. Recently some reports proved that solid state fermentation (SSF) is highly effective in the production of lipase [5].

SSF is a promising tool in biotechnology field for the production of microbial metabolites through inexpensive means and it is the most appropriate process for developing countries [6]. SSF has numerous advantages over conventional submerged fermentation, including resembling the natural habitat for several microorganisms, lower-cost media, better oxygen circulation, reduced energy and cost requirements, less operational problems, the less effect in downstream processing, higher productivity, compactness of fermentation vessel, lower capital and recurring expenditure etc. [7] optimized the synthesis of lipase by the yeast *Candida rugosa* through SSF and found the C: N ratio of the medium to be an important parameter for lipase activity. The production of lipase by *Penicillium candidum* in submerged (SF) and solid state fermentations, verifying the superiority of SSF processes [8]. Bacterial lipases are mostly released outside of the cell that is called extracellular enzyme. In order to improve the whole-cell lipase catalytic ability in organic solvent, the effects on synthetic enzyme production must be studied systematically and the culture condition needs to be optimized [9]. Improvement of lipase production still depends on the optimization of culture conditions, including the composition of the culture medium such as carbon and nitrogen sources and other fermentation parameters such as dissolved oxygen, temperature and aeration rate [10]. Agro- industrial residues such as olive oil cake, soy cake, babassu cake, wheat bran and almond meal were used as substrates for the production of lipases by SSF [11]. Mixed solid substrates such as coconut oil cake: wheat bran (1:1) on lipase production was also studied [12]. Microbial lipolysis has been studied frequently, but very little work related to this has included in studies with chemically defined media [13]. Downstream processing is a fundamental for any fermentation process and it involves isolation and purification sequences to obtain a pure and homogenous product like enzymes [14]. Chartrain [15] reported that, the lipase produced by *Pseudomonas aeruginosa* MB5001 was purified by using three-step procedure, which include concentration by ultrafiltration, followed by ion exchange chromatography and gel filtration. This purified lipase had a molecular mass of 29 kDa by SDS-PAGE. Lee and Rhee [16] Inferred that, the lipase of *P. putida* 35K was purified by ion exchange and gel filtration chromatography systems, however its activity or stability was inhibited by mercury ions and SDS. Based on the above mentioned factors Considering the importance of microbial lipase, the present work was made to evaluate the lipase production using onion skin waste as the major substrate in optimized culture conditions by *Bacillus cereus* MSU AS, an isolate from the gut of a marine fish *Sardinella longiceps* through solid state fermentation processes.

MATERIALS AND METHODS

Selection of Bacterium and lipase activity

The bacterium used in this study was isolated from the gut of a marine fish *Sardinella longiceps* collected from Colachal coast of Kanniyakumari District, Tamil Nadu, India. The bacterium produced a clear zone when it was streaked on sprit blue agar plates supplemented with glycerol tributyrin. The candidate bacterial strain was identified as *Bacillus cereus* MSU AS based on the standard key's of Bergey's manual of determinative bacteriology and molecular characterization (16S rRNA). The 16S rRNA sequence of *Bacillus cereus* MSU AS was compared with other similar bacterial groups by NCBI- BLAST data base program and then it was deposited in NCBI data bank (Accession no: JF-907013).

Screening of various agricultural waste substrates on lipase production by the candidate bacterium through SSF

For lipase production under solid state fermentation, various agricultural waste substrates were used. To 10ml of basal medium (Glucose: 1g, KH_2PO_4: 0.1g, $MgSO_4$: 0.02g, NaCl: 1g) and 5g each of agricultural wastes such as coconut oil cake (COC), neem oil cake

(NOC), onion skin waste (OSW), wheat bran (WB), jack fruit waste (JFW), banana skin peel (BSP) and pomegranate skin peel (PSP) were added individually. Then in the sterilized mixed basal medium, 2ml of seed culture of *B. cereus* MSU AS was inoculated, and allowed for incubation at 37°C for 48h at room temperature. The effect of the individual supplemented substrates on lipase production was determined by lipase assay. From this, the maximum lipase yielding substrate was selected for further studies.

Solid–State Fermentation (SSF)

From the above screening study on lipase production, onion skin supplemented medium showed a maximum yield of lipase, therefore it was used as a major substrate for further SSF studies. For this, *B. cereus* MSU AS was enriched using enrichment medium (Peptone - 0.05g, NaCl - 0.55g, Yeast extract - 0.02g, Beef extract - 0.01g, pH - 7.4 in 50ml of distilled water). The medium was sterilized at 121°C for 15 min and then inoculated with the candidate strain. The flask was incubated for 24h at 37ºC on a rotary shaker at 150rpm. Then 2ml of enriched seed culture was inoculated in 250ml Erlenmeyer flask containing sterilized 10ml mineral medium (Glucose - 0.1g, KH_2PO_4 - 0.01g, $MgSO_4$ - 0.002g, NaCl - 0.1g and pH - 7.2 in 10ml of distilled water) along with 5g of pre sterilized solid onion skin waste (OSW). The culture was then incubated for 48h. After incubation, 50ml of distilled water was added and placed in a shaker at 150 rpm for 1h [17]. Then the cells were filtered by using filter paper and then centrifuged at 10,000 rpm for 15min and the supernatant was used for the lipase assay.

Lipase activity was quantified according to the modified method of [18]. The lipase assay system consists of the following ingredients: 1ml Tris buffer (pH 7.2), 1ml p-nitro phenyl palmitate (10mM) and 0.5 ml culture supernatant. Approximate controls were also maintained. The mixture was incubated for 15 min on a rotary shaker at 150 rpm at 37°C. Then the absorbance was measured at 400nm using UV-Vis Spectrophotometer (Techomp - 8500), the amount of lipase produced was determined with the help of p-nitro phenol standard graph. One unit of lipase activity is equivalent to one microgram of p-nitro phenol released under standard assay condition.

Lipase production in various culture conditions and fermentation parameters

The lipase production by *B. cereus* MSU AS was optimized through different parameters such as moisture, carbon source, nitrogen source, NaCl and triglycerides (inducer).

Effect of different volume of moisture content on lipase production

The influence of moisture on lipase production during SSF was determined. For this, the selected substrate was moistened with different amount of liquid media (10, 15, 20, 25, 30, 35, 40, 45, 50, and 55%) by adding sterilized water. Then 2ml each of the seed culture was inoculated in the respective medium. The lipase production was measured after 48h interval of incubation.

Effect of various carbon sources on lipase production

To study the effect of various carbon sources on lipase production by the candidate bacterium, nine different carbon sources such as glucose, sucrose, fructose, maltose, lactose, xylose, sorbitol, galactose and mannitol were screened. They were tested individually into mineral medium at the concentration of 0.2% and the control was assayed without carbon sources. The effect was determined after 48h of incubation. After that the carbon source giving maximum positive effect on lipase production was optimized through varying its concentrations (0.2, 0.3, 0.4, 0.5 and 0.6%).

Effect of nitrogen sources on lipase production

For the selection of suitable nitrogen sources on lipase production by the candidate species under SSF, eleven different organic and inorganic nitrogen sources were screened. They were: beef extract, peptone, sodium nitrate, ammonium chloride, ammonium nitrate, yeast extract, soya meal, skim milk powder, ammonium hydrogen carbonate, potassium nitrate and ammonium sulphate. They were supplied individually at the concentration of 0.1% into the mineral medium. The medium without nitrogen source was taken as control. The effect of these nutrients was determined after 48h of incubation. After that the maximum lipase producing nitrogen source was optimized for lipase production through varying its concentrations (0.1, 0. 2, 0.3, 0.4, 0.5 and 0.6%).

Effect of different concentrations of NaCl on lipase production

As the lipase producing *B. cereus* MSU AS was isolated from the gut of a marine fish *S. longiceps*, therefore the NaCl was essential to maintain its osmo regulation. Due to its importance, various concentrations of NaCl (1, 2, 3, 4, 5, 6, 7, 8 and 9%) were tested for lipase production. They were supplied individually in the mineral medium and the effect of NaCl on lipase production was determined after 48h of fermentation.

Effect of different triglycerides on lipase production

Lipase is often inducible by triglycerides; therefore the effect of different oils on lipase production by the test organism was estimated. The lipids tested were gingilly oil, olive oil, neem oil, coconut oil, castor oil, cod liver oil, palm oil and sunflower oil. They were supplied individually in mineral medium at the volume of 0.1%; the medium without any of the above lipid was taken as control. After 48h of incubation, the assay was done. The triglyceride giving maximum positive effect on lipase production was further optimized through varying its volume (0.1, 0.2, 0.3, 0.4, and 0.5%).

Purification of lipase through ammonium sulphate precipitation and chromatography methods

The organism was cultured at 37°C in a optimized basal medium consisting of moisture content – 10%, maltose -0.5%, ammonium hydrogen carbonate – 0.5%, gingilly oil - 0.4% and NaCl – 9%. Then this medium was sterilized at 121ºC for 15 min. After that, 2 ml of seed culture of *B. cereus* MSU AS was inoculated and incubated for 48h. After 48 h of fermentation *B. cereus* culture broth was filtered on Whatman paper No. 1 to eliminate all biomass. Benzamidine, a serine protease inhibitor was added to the culture broth to a final concentration of 2mM to prevent the occurrence of proteolytic degradation during the purification procedure. Lipase extraction from the solid fermentation medium was carried out by adding 125 ml of 20mM Tris–HCl buffer (pH 7.2) containing 0.5% Triton X-100 and 2mM benzamidine. The filterate was centrifuged at 10,000 rpm for 30 min at 4ºC. After centrifugation, collected the supernatant and discarded the pellet, then allowed for precipitation with 75% ammonium sulphate fractionation. After that, the precipitated sample was dissolved in 1 ml Tris-HCl buffer and dialyzed (10 KDa dialysis membrane) overnight against 4 L of the 5mMTris-HCl buffer. Then the fraction was checked for enzyme activity as well as protein content further 15 ml of the fraction was applied into a preactivated DEAE cellulose column (column length: 35 cm and packing length:15 cm).

The enzyme was eluted with linear gradient of 50ml of 5 mM Tris-HCl buffer (pH 7.2), and also equilibrated with different concentrations of NaCl solution within the range of 0.05, 0.25, 0.50, 0.75 and 1M with the flow rate of 5ml/min. All the fractions were checked for enzyme activity. The active fractions were pooled and applied on Sephadex G-75 for separation of protein based on the size of the molecules. Sephadex G -75 column and equilibrated with 75 ml of 0.05M sodium phosphate buffer (pH 7). At 2 min intervals, 5 ml each of individual fractions were collected. Then each fraction was tested for lipase and protein contents through Spectrophotometric assay method [19]. The protein content at each stage of enzyme purification was determined according to the method of [20] with Bovine serum albumin as the standard.

Determination of molecular weight of protein and lipase activity by SDS and Native PAGE

Molecular weight of lipase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide as described by [21]. A low molecular mass marker kit (Amersham) was used to determine the molecular mass. The gels were stained with Coomassie brilliant blue R-250 to reveal the proteins.

Lipase active compound was determined by Non-denature polyacrylamide gel electrophoresis [22]. After electrophoresis, the gel was taken out from two glass plates with the help of distilled water and placed into the shallow tank (50 ml Trise –HCl buffer, pH 7.2, 0.5 ml of 0.1 M $CaCl_2$ and1 ml of glycerol tributyrin) for 1h. After that, the gel slap was immersed in Coomassie colloidal blue staining solution tank until showed the lipase active compound, it indicated the zone of inhibition at the presence of lipase active protein present area.

Statistical Analysis

The data obtained in the present study were expressed as Mean ± SD and were analysed using One-way ANOVA test at 5% significant level. Further multiple comparision test (SNK) was conducted to compare the significant differences among the parameters using computer software statistica 6.0 (Statosoft, UK).

RESULTS

Bacterium and lipase activity

In the present study, lipase producing bacterial strain was isolated from the gut of a marine fish *S. longiceps* and then it was screened for its lipase producing ability on spirit blue agar. From the result, it was found that, the bacterial strain produced a clear zone of lipase production (17 mm) after 48h of incubation and it was due to the hydrolysis of tributyrin (Figure 1).

This lipase positive strain was subjected to conventional method of identification based on its morphological, physiological and biochemical characteristics, and it was identified as *Bacillus* sp. Further, the BLAST research on the 16S rRNA sequence of the candidate strain showed 97% similarity to *Bacillus cereus* strain L12 (HQ398861). The sequence of the candidate bacterial strain *Bacillus cereus* MSU AS was submitted to GenBank in NCBI data base under the accession no JF907013 (Figure.2).

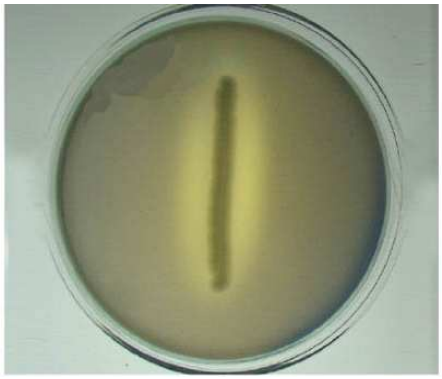

Fig. 1: Primary screening of lipase producing gut bacterial isolate in spirit blue agar medium.

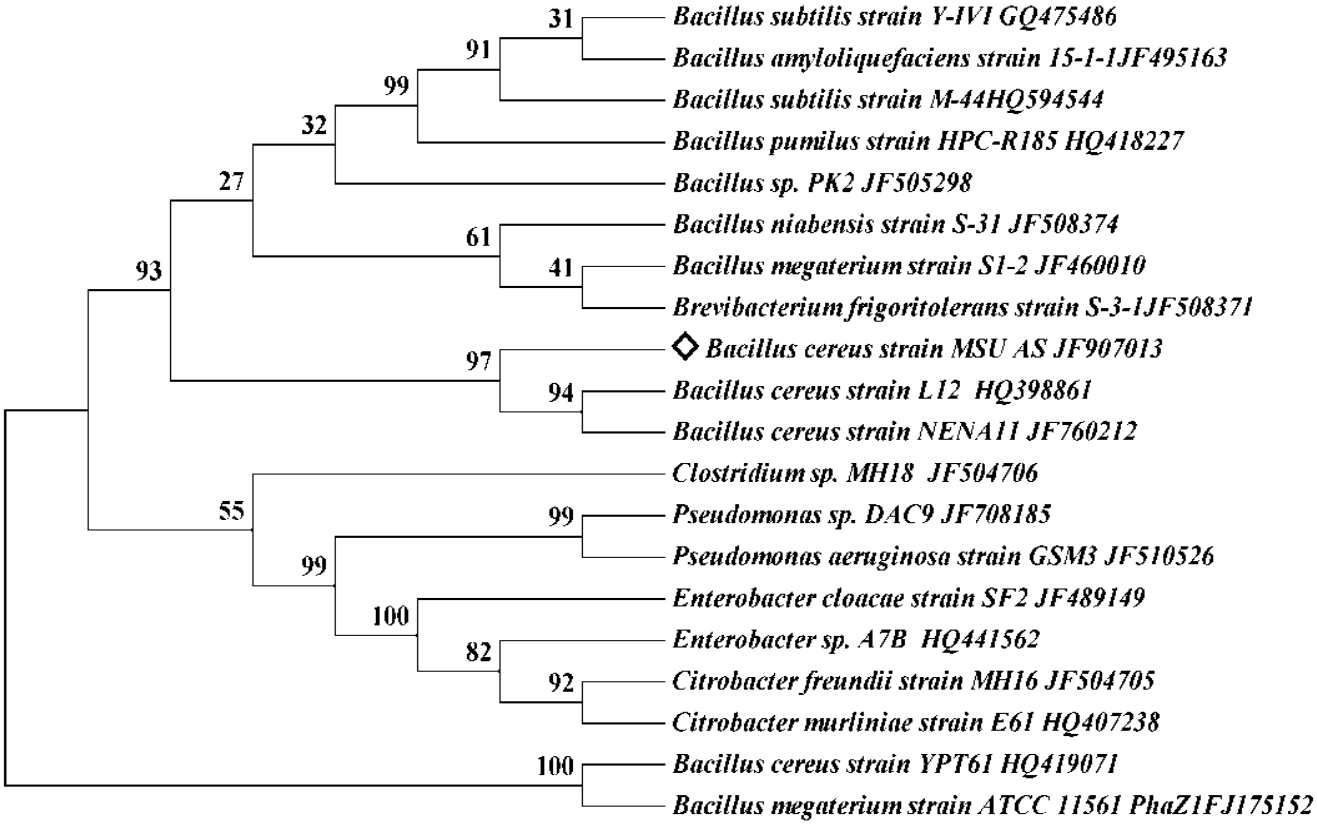

Fig. 2: Phylogenetic relationship of lipase producing bacterium *Bacillus cereus* strain MSU AS by using 16S r RNA.

◇: Candidate bacterium (*Bacillus cereus* strain MSU AS)

Screening of various agricultural waste substrates on lipase production by the *B. cereus* MSU AS through SSF

In the present study, seven different cheapest agricultural waste substrates such as coconut oil cake, neem oil cake, onion skin waste, wheat bran, jack fruit waste, banana skin waste and pomegranate peel were screened for their suitability on lipase production under SSF by *B. cereus* MSU AS. Among the tested substrates, onion skin waste yielded maximum (873.84 U/g) lipase; whereas banana skin waste recorded very low amount (31.13 U/g) of lipase production (Figure 3). Based on this result, the maximum lipase producing substrate onion skin waste was selected for further optimization studies.

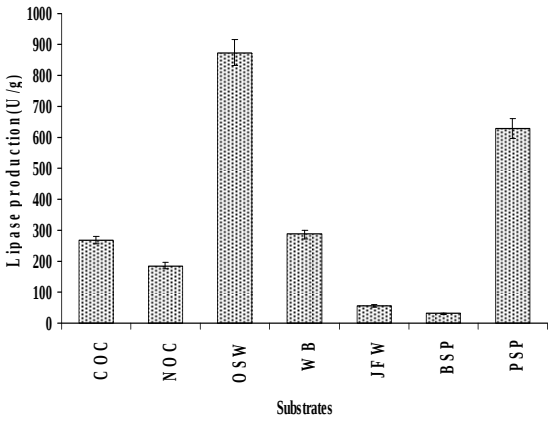

Fig. 3: Influence of different agricultural waste substrates on solid state fermentation for lipase production by *B. cereus* MSU AS.

Effect of different volume of moisture content on lipase production

To determine the optimum level of moisture content, in the present study, different levels (10-55%) of moisture content was employed. From the result, it was understood that 1:1 (10%) ratio of substrate: water (w/v) was more favorable for maximum (406.69 U/g) lipase production by *B. cereus* MSU AS, further increase in level of moisture content lead to decrease in level of lipase production (Figure 4). The one way ANOVA for the data on lipase production as a function of variation due to different moisture level was statistically more significant (F = 1792914; P < 0.0001).

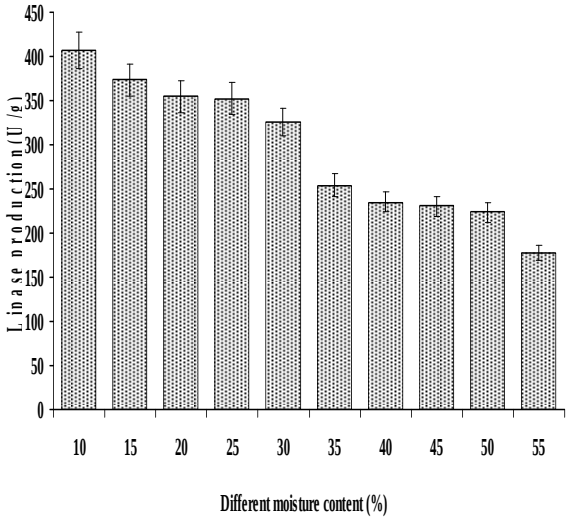

Fig. 4: Effect of different moisture content on lipase production by *B. cereus* MSU AS.

Effect of various carbon sources on lipase production

The result on the ability of *B. cereus* MSU AS on lipase production by utilizing the carbon sources is given in Table 1. Among the tested carbon sources, maltose recorded the highest (350.19 U/g) influence on lipase production. The other carbon sources such as mannitol (346.24 U/g), sucrose (343.18 U/g) and galactose (323.54 U/g) gave moderate result on lipase production. The one way ANOVA for the data on lipase production as a function of variation due to different carbon sources was statistically more significant (F = 240.8322; P < 0.0001). Further the lipase production at various concentrations of maltose revealed that 0.5% concentration favor the maximum (357.76 U/g) lipase production (Figure 5).

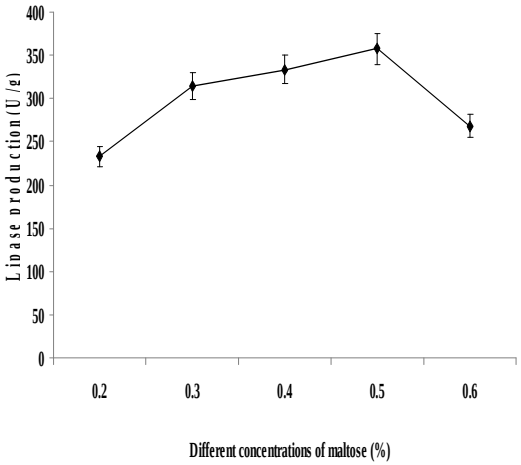

Fig. 5: Effect of different concentrations of maltose on lipase production by *B. cereus* MSU AS.

Effect of nitrogen sources on lipase production

The effect of different nitrogen sources on lipase production by *B. cereus* MSU AS revealed that highest (407.85 U/g) lipase production was observed in inorganic nitrogen source ammonium hydrogen carbonate supplemented medium and skim milk powder gave minimum (88.28 U/g) lipase production (Table 1). The one way ANOVA test conducted for the data on lipase production as a function of variation due to different nitrogen sources was statistically significant (F = 3699.972; P < 0.0001). The result on different concentrations of ammonium hydrogen carbonate showed that 0.5% concentration was found to be an optimum to produce maximum (605.97 U/g) amount of lipase (Figure 6).

Effect of different concentrations of NaCl on lipase production

The effect of different concentrations of NaCl on lipase production by *B. cereus* MSU AS revealed that the optimum amount of lipase (402.16 U/g) production was recorded at 9% NaCl supplemented medium (Table 1). The statistical one way ANOVA for the data on lipase production as a function of variation due to different concentrations of NaCl was more significant (F = 1506.304; P < 0.0001).

Effect of different triglycerides on lipase production

Triglycerides are found to be either inducer or inhibitor for lipase or esterase production. In the present study, eight different inducers such as olive oil, gingilly oil, neem oil, coconut oil, castor oil, cod liver oil, sunflower oil and palm oil were individually screened for their ability to support lipase production (Figure 7). Results indicated that among the tested triglycerides, gingilly oil supported maximum (326.03 U/g) lipase production by *B. cereus* MSU AS, followed by caster oil (303.34 U/g), which was also supported for maximum lipase production. The one way ANOVA for the data on lipase production as a function of variation due to different triglycerides was statistically more significant (F = 1679.209 P < 0.0001). Since, gingilly oil contributed more lipase production; further the effect of different concentrations of it was tested for lipase production. It resulted that 0.4% gingilly oil was found to influence maximum lipase production by *B. cereus* MSU AS (Figure 8).

Table 1: Effect of different carbon sources, nitrogen sources and different concentrations of NaCl on lipase production by *B. cereus* MSU AS

Carbon sources	Lipase production (U/g)	Nitrogen sources	Lipase production (U/g)	Different NaCl (%)	Lipase production (U/g)
Control	$324.74^{a} \pm 2.449$	Control	$285.3^{a} \pm 2.286$	Control	$212.06^{a} \pm 2.420$
Glucose	$294.07^{b} \pm 2.122$	Yeast extract	$67.34^{b} \pm 1.012$	1	$238.91^{b} \pm 3.404$
Fructose	$298.30^{b} \pm 3.204$	Soya meal	$128.81^{c} \pm 2.122$	2	$247.85^{bc} \pm 2.420$
Lactose	$306.79^{b} \pm 2.286$	Beef extract	$237.11^{d} \pm 2.204$	3	$266.02^{d} \pm 2.206$
Maltose	$350.19^{c} \pm 2.367$	Skim milk powder	$88.28^{e} \pm 2.020$	4	$338.34^{e} \pm 3.286$
Xylose	$299.36^{bd} \pm 3.206$	Peptone	$262.79^{f} \pm 3.286$	5	$342.08^{ef} \pm 3.367$
Mannitol	$346.24^{ce} \pm 4.268$	$NaNO_3$	$214.32^{g} \pm 2.620$	6	$356.16^{fg} \pm 3.449$
Sorbitol	$254.54^{f} \pm 2.142$	NH_4Cl_2	$206.94^{gh} \pm 2.046$	7	$372.26^{gh} \pm 2.531$
Sucrose	$343.18^{ceg} \pm 2.280$	NH_4NO_3	$256.29^{fi} \pm 3.367$	8	$395.18^{i} \pm 3.612$
Galactose	$323.54^{ah} \pm 4.367$	NH_4HCO_3	$407.85^{j} \pm 4.612$	9	$402.16^{ij} \pm 4.694$
-	-	KNO_3	$160.77^{k} \pm 2.182$	-	-
-	-	NH_4SO_4	$214.32^{ghl} \pm 2.204$	-	-

Each value is the Mean ± SD of the three estimates. Within each column, means with the different superscript letters are statistically significant (One – way ANOVA test, $p < 0.0001$ and subsequently *post hoc* multiple comparison with SNK test)

Table 2: Purification summary of lipase produced by *Bacillus cereus* MSU AS

	Total activity of lipase (U)	Lipase activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Yield of lipase (%)	Purification fold of lipase (%)
Crude lipase	101004.0 ± 186.46	219.0 ± 4.63	1.21 ± 0.04	180.9 ± 4.36	100 ± 1.63	-
Ammonium sulphate (75%) precipitation	64061.0 ± 74.28	1389.0 ± 38.44	3.20 ± 0.21	434.0 ± 6.84	63.0 ± 4.0	2.5 ± 0.040
Dialyzed sample	73239.0 ± 96.42	1588.0 ± 32.36	0.24 ± 0.022	1312.3 ± 24.26	72.0 ± 6.0	7.25 ± 0.68
DEAE-Cellulose column	85184.0 ± 78.36	1847.0 ± 48.82	0.20 ± 0.012	9235.0 ± 18.32	84.0 ± 6.40	51.0 ± 3.66
Sephadex G-75 chromatography	89427.0 ± 69.46	1956.0 ± 26.24	0.16 ± 0.002	12225.0 ± 98.74	89.0 ± 3.0	67.0 ± 4.0

E ach value is the Mean ± SD triplicate analysis

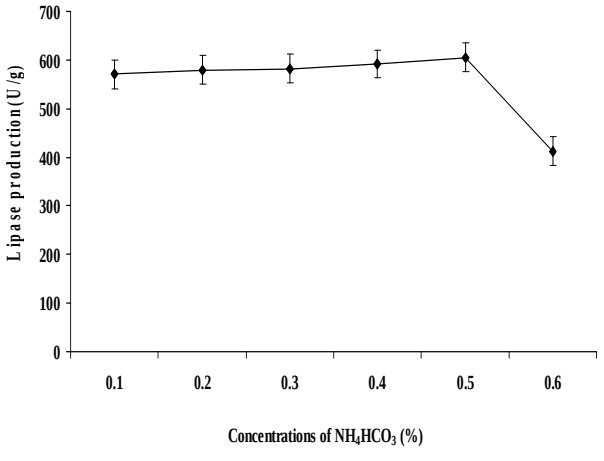

Fig. 6: Effect of different concentrations of NH_4HCO_3 on lipase production by *B. cereus* MSU AS.

Purification of lipase through ammonium sulphate precipitation and chromatography methods

The lipase produced by *B. cereus* MSU AS in the culture broth was subjected to a purification protocol. The lipase activity of crude filtrate was 219.0 ± 4.63 U/ml and its corresponding specific activity was 180.9 ± 4.36 U/mg (Table 2). Protein was precipitated by ammonium sulphate precipitation method and it was separated by centrifugation under reduced temperature. The lipase activity of 1389.0 ± 38.44 U/ml and protein content of 3.20 ± 0.21 mg/ml were measured in the supernatant after 75% ammonium sulphate saturation. Therefore this pellet was further dialyzed. The dialyzed pellet displayed the maximum lipase activity of 1588.0 ± 32.36 U/ml, total activity of 73239.0 ± 96.42 U, total protein content of 0.24 ± 0.022 mg/ml, specific activity of 1312.3 ± 24.26 U/mg, purification fold of 7.25 ± 0.68 % and lipase yield of 72%, when compared to that of the pellet obtained in ammonium sulphate precipitation method (Table 2).

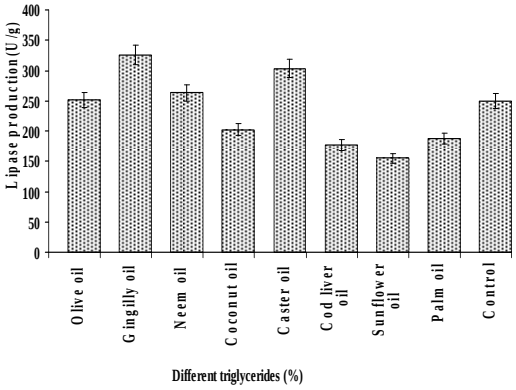

Fig. 7: Effect of different triglycerides on lipase production by *B. cereus* MSU AS.

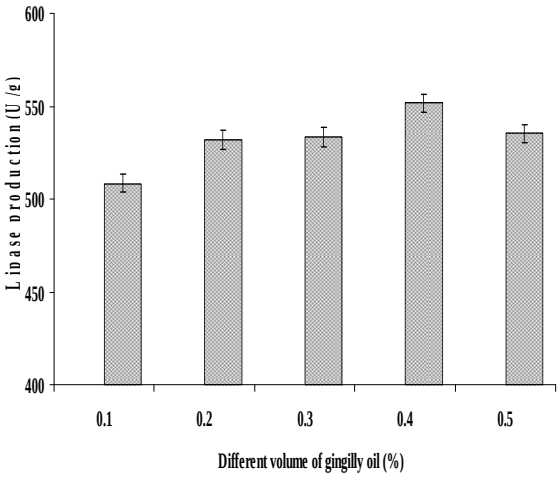

Fig. 8: Effect of different volume of gingilly oil on lipase production by *B. cereus* MSU AS.

The dialyzed sample was further purified through DEAE –cellulose column process. Here maximum lipase activity of 1847.0 ± 48.82 U/ml and protein content of 0.20 ± 0.012 mg/ml were observed in fraction 2 (elution) of 0.75 M NaCl concentration. Its corresponding specific activity, purification fold of lipase and yield of lipase were obtained respectively as 9235.0 ± 18.32 U/mg, 51.0 ± 3.66% and 84.0 ± 6.46%. The final step of lipase purification through Sephadex G-75 column indicated that the fraction 9 gave maximum lipase activity (1956.0 ± 26.24 U/ml) and protein content (0.16 ± 0.002 mg/ml). Its corresponding specific activity was 12225.0 ± 98.74 U/mg, purification fold of lipase was 67.0 ± 4.0%, and the yield of lipase was 89.0 ± 3.0% (Table 2).

Determination of molecular weight of protein and lipase activity by SDS and Native PAGE

The purity of lipase was checked by using SDS-PAGE with standard markers as mentioned earlier and the molecular weight of purified lipase of *B. cereus* MSU AS recorded was 49kDa (Figure 9). Lipolytic active protein of *B. cereus* MSU AS (49kDa) was also detected and confirmed through Native PAGE analysis of the sample of Sephadex G-75 column fraction (Figure 10).

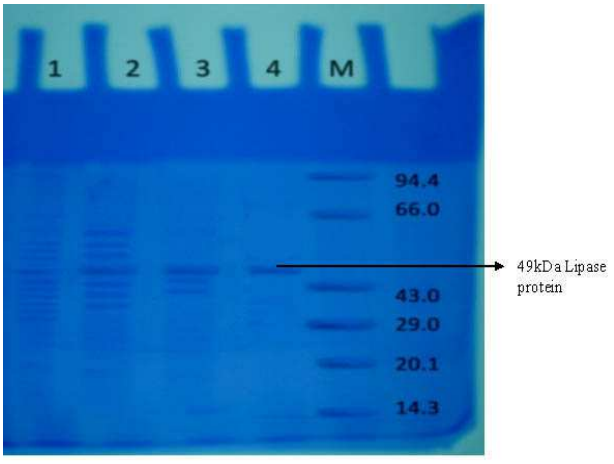

Fig. 9: SDS-PAGE pattern of purified lipase.. It was conducted in 10% gel. Lane 1: Crude sample, Lane 2: 75% ammonium sulphate precipitated sample, Lane 3: DEAE –Cellulose fraction sample; Lane 4: 49kDa Purified lipase from Sephadex G- 75 column fraction; Lane M: Protein molecular weight markers.

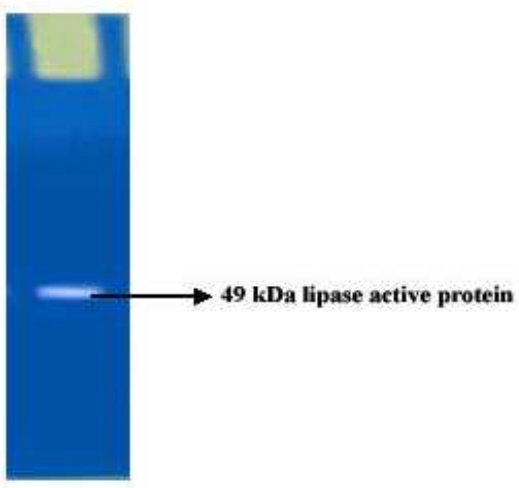

Fig.10: Native-PAGE pattern of purified lipase. It was conducted in 10 % gel. Well showing clear zone indicated for 49 kDa lipase activity from Sephadex G- 75 column fraction sample

DISCUSSION

Gut microbial enzymes play an important role in conversion of complex substances in to simpler substances. Among various enzymes, protease, lipase, amylase and cellulose acquire much attention. In the present study, gastrointestinal bacterial strains were isolated from the gut of a marine fish *S. longiceps* and their lipase producing ability was also evaluated. Sivasubramanian [23] evidenced that, the enzymes such as amylase, protease and lipase producing *Acinetobacter, Bacillus, Enterobacteriaceae, Vibrio, Alcaligenes, Photobacterium, Pseudomonas, Aeromonas* and *Flavobacterium* spp. were isolated from the gut of fishes like *Oreochromis leucostictus, O. mossambicus, Etroplus suratenis,* etc. Lipase activity can be identified by using triacylglycerols composed of long-chain fatty acids. Substrates like tweens and tributyrin can also be used for the detection of lipases [24]. In the present investigation also spirit blue agar medium supplied with 1% glycerol tributyrin was used for primary screening of lipase production by the test organisms. Likewise, Bruni [25] confirmed the strong lipolytic activity of *Pseudomonas* sp. NCMB 1082 by using Tween and tributyrin in the spirit blue agar medium.

Molecular identification (16S rRNA sequencing) of bacterial strain up to species level is one of the most advanced and excellent alternatives to conventional or classical identification system. In the present study, the maximum lipase producing strain was subjected to morphological and various biochemical studies and identified up to genus level as *Bacillus* sp. Thereafter, the 16S rRNA sequencing of *Bacillus* sp. showed 97% similarity to *B. cereus* strain L12 (HQ398861). Thus the candidate bacterial strain was designated as *B. cereus* MSU AS and it was submitted to GenBank in NCBI data base under the accession number JF 907013. Similarly, Dutta and Ray [26] identified the alkaline thermostable lipase producing strain of *B. cereus* C7 (AB 24464) through 16S rRNA sequencing analysis, and this strain showed 96% similarity with *B. cereus* St. NK1 (AB 295052). Recently some reports on the production of lipase by solid state fermentation (SSF) were published. Ohnishi [27] studied the production of lipase by *Aspergillus oryzae* with different solid substrates. The use of cheap raw materials would diminish the operating costs of the fermentation process. Similarly, Ul-Haq *et al.* [28] investigated various agro products such as wheat bran, rice bran and wheat husk to whole cell synthetic lipase (WCSL) production by *Rhizopus chinensis* CCTCCM201021, and they pointed out that wheat husk served as the best substrate for maximum lipase production (1500 U/kg). In the present study, seven different cheaper agricultural waste substrates were screened for lipase production. Among the substrates tested, onion skin waste yielded maximum lipase production (873.84 U/g) by *B. cereus* MSU AS.

Solid state fermentation (SSF) is defined as the fermentation of solids in the absence of free water; however, the substrate must possess enough moisture to support the growth and metabolism of microorganisms. In the present study the maximum lipase production and showed in 10% moisture content of lipase production medium. In the same way, Balaji and Ebenezer, [29] tested the effect of moisture content on lipase production by *Aspergillus niger* and inferred the maximum lipase production at 15% moisture content. Comparing the findings of different authors, it can be concluded that the effect of moisture content on lipase production may be varied depends on the types of organisms.

Carbon source is an important substrate for energy production in microorganisms. In order to investigate the effect of carbon sources on lipase production by *Staphylococcus* sp. Lp12, Pogaku *et al.* [30] screened a range of carbon sources for their efficiency to support lipase production. Rao *et al.* [31] found that the *Candida rugosa* influenced maximum on lipase production, when it was cultured at 0.5% of maltose supplemented medium. In accordance to this, in the present study also, it was observed that the maximum lipase production was occured in 0.5% maltose supplemented medium.

The type of nitrogen source in the medium also influences the lipase titers in production broth. Generally, organic nitrogen sources, such as peptone and yeast extract are preferred, which have been used as a major source for lipase production by various *Bacillus* sp. In the present study, the maximum lipase was produced in 0.5% ammonium hydrogen carbonate supplemented medium by *B. cereus* MSU AS. Invariably, Thomas *et al.* [32] have pointed out that beef extract was the best nitrogen source, followed by yeast extract and peptone for lipase production by *B. mycoides*.

In the present study, it was observed that maximum lipase production by *B. cereus* MSU AS in the medium supplemented with 9% NaCl. This was in accordance to the result of Ventosa *et al.* [33], who found significantly increased lipase production by halotolerant strain of *B. pumilus* with respect to increase in NaCl concentration from 5 to 10% and also they pointed out that this behavior might involve the fact that this is a salt dependent enzyme, either for stability or activity of the enzyme.

The present result indicated that among the tested triglycerides, gingilly oil supported maximum (326.03 U/g) lipase production by *B. cereus* MSU AS. In agreement with the present finding, Immanuel *et al.* [34] stated that lipase production by *Serratia rubidaea* was highly influenced by 0.5% gingilly oil. In the present study, *B. cereus* MSU AS produced more lipase in 0.4% gingilly oil supplemented medium.

Purification of enzyme sources, has a restricted capacity to improve the specific activity, reaching a maximum of 5 times than the initial value. In the present study, crude *B. cereus* MSU AS lipase was obtained with 2.5% purification fold in 75% ammonium sulphate precipitation processes. Likewise, Weerasooriya and Kumarasinghe [35] documented that the rubber seed lipase was observed 2.84% fold purification with 85.16% recovery by the saturation of 75% ammonium sulphate. In the present study after ammonium sulphate precipitation, the *B. cereus* MSU AS lipase fraction with 2.5% purification fold was allowed for dialysis process. After dialysis process, the purity of lipase was checked and it was recorded with 7.25% purification fold. Further this desalting sample was allowed for ultrafiltration by ion exchange chromatography using DEAE – cellulose. The DEAE – cellulose lipase positive fraction was obtained with 51% purification fold lipase and 9235.0 U/mg of specific activity. In accordance with these, Iftikhar *et al.* [36] stated that, the lipase produced by *Rhizopus oligosporus* var. *microsporus* showed 2.34% fold purification and 77.01% of product yield after desalting process. In the present study after ion exchange chromatography purification, the 51% purification fold lipase of *B. cereus* MSU AS was further filtered with the help of size exclusion chromatography of Sephadex G-75 and it resulted 67% fold purified lipase. Likewise, Zheng –Yu *et al.* [37] documented that the lipase produced by *Aspergillus niger* F044 was allowed for purification by Sephadex G-75, it resulted with 73.71% fold purification and 33.99% yield. In the present study, after gel filtrations on Sephadex G-75 column purification, the 67% purification fold lipase was subjected to 12% SDS PAGE and the molecular weight of lipase protein produced by *B. cereus* MSU AS was estimated as 49kDa. In accordance with these, Hiol *et al.* [22] also stated that the lipase produced by *M. hiemalis f. hiemalis* was subjected to filtrations through Sephadex G-75 column, and the molecular weight of this purified lipase was estimated to be 49 kDa by SDS PAGE.

CONCLUSION

In the present study, an attempt was made to isolate and identify a novel lipase producing bacterium *B. cereus* MSU AS from the gut of a marine fish *S. longiceps*. The lipase production by the candidate bacterial strain through SSF was found to be accelerated at optimized culture conditions such as moisture and various substrates concentrations. From the result it could be concluded that, the optimum substrates required for enhancing production of lipase were 0.5% maltose, 0.5% ammonium hydrogen carbonate, 9% NaCl and 0.4% gingilly oil. Further the lipase produced by *B. cereus* was purified and the molecular weight of this purified lipase was determined as 49 kDa. Through this study, it could be confirmed that *B. cereus* is a potential strain for lipase production by utilizing the cheapest substrate onion skin waste through SSF.

CONFLICT OF INTEREST: Nil

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