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Research Article

Evaluation of Production Parameters for Maximum Lipase Production by *P. stutzeri* MTCC 5618 and Scale-Up in Bioreactor

Vishal Thakur, R. Tewari, and Rohit Sharma

Department of Microbial Biotechnology, Panjab University, Chandigarh 160014, India

Correspondence should be addressed to Rohit Sharma; rohit28@yahoo.com

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Intracellular lipase producer screened from the library available in the laboratory, identified through 16S rRNA as *Pseudomonas stutzeri*, was studied for maximum enzyme production in shake flask. The work was intended to evaluate the effect of different physicochemical factors like carbon, nitrogen, metal ions, surfactant, inoculum, pH, temperature, agitation, and aeration on lipase production. Optimized media showed 1.62-fold increase in lipase production when compared to basal media. Scale-up of lipase in *in situ* bioreactor showed reduction in fermentation time in both basal and optimized media, giving 41 and 99 U/mg of lipase activity after 48 h of fermentation.

1. Introduction

Lipases are carboxylesterases (E.C. 3.1.1.3) that catalyze both hydrolysis and synthesis of esters formed from glycerol and fatty acids [1, 2]. They are the serine hydrolases which generally act at the oil-water interface. Structural attributes include an α/β hydrolase fold and the active site of the enzyme is generally composed of catalytic triad which is surrounded by a consensus sequence of Gly-x-Ser-x-Gly [3]. Lipases are currently attracting an enormous attention because of their wide range of applications in natural sciences and biotechnology [4]. They often retain their biological activity in organic solvents and display exquisite chemo-, regio-, and enantioselectivity that provide them an edge over other classes of biocatalysts. Due to their properties they have become almost indispensable biocatalysts in various industrial sectors such as the agrochemical, pharmaceutical, detergent, and food industries [5]. The number of lipases has increased since the 1980s and they are studied from microorganisms such as *Pseudomonas, Bacillus, Achromobacter, Alcaligenes, Arthrobacter,* and *Chromobacterium* [6–8]. It is a well known fact that variation in media components has a significant role in influencing microbial metabolic activities and thereby enzyme production. Generally, high enzyme titre has been achieved by culture medium changes in terms of various possible physicochemical components that are likely to influence the enzyme production and growth of microorganisms in time course fermentation [9]. Optimization and evaluation of each ingredient that constitutes a cultivation medium is the foundation in classical approach. Therefore optimizing best possible conditions for maximum production of enzyme is still an important and imperative area in enzymology.

In the present study, lipase producer *Pseudomonas stutzeri* MTCC 5618 was studied for maximum enzyme production through media engineering approach. The influence of different physicochemical factors on intracellular lipase production in time course shake flask fermentation was studied and scale-up for enzyme production in 5 L *in situ* bioreactor was also carried out.

2. Material and Methods

2.1. Chemicals and Reagents. *p*-Nitrophenyl butyrate (*p*-NPB) was obtained from Sigma Aldrich, St. Louis, MO, USA, methanol was obtained from Merck Specialities Pvt. Ltd., Mumbai, India, and the media were purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, India.

Instruments used in the present work were from standard companies. Autoclave used was from Narang Scientific Works Pvt. Ltd., New Delhi, India. Bioreactor (Biospin Lab Scale L Series Model 1-Biospin-05A) used was from Bio-Age Equipment and Services, Mohali, India, cooling centrifuge (Model number C-30 BL), shaker (Model number CIS-24 BL) were of Remi Elektrotechnik, Ltd., Mumbai, India, and microscope (Model number CX21i) was from Olympus Opto Systems Pvt. Ltd., Noida, India. Spectrophotometer (Model number 1372) and pH meter (Model number 962-P) used were from Electronics India, Parwanoo, India, and weighing balance used was from (Denver S1-234, Denver MXX 212) Denver Instrument GmbH, Goettingen, Germany.

2.2. Microorganism. The isolate RV68 used in the present study was screened from the library available in laboratory and identified through 16S rRNA technique. Culture was maintained on nutrient agar plate and tested for lipase production on selective plate containing tributyrin (TB) (4.2 mM) supplemented with methanol (10% v/v).

Selection of the isolate was made on the basis of colony : zone (C : Z) diameter ratio when grown on tributyrin agar plate incubated at 30°C for different intervals of 24, 48, and 72 h. Secondary screening included study for growth and enzyme production in 250 mL Erlenmeyer flask containing 50 mL production media, including 1.3% (w/v) nutrient broth and 4.2 mM, 8.4 mM, and 12.6 mM TB, respectively. Production media were inoculated with 2% (v/v) bacterial seed culture (absorbance $A_{600\,nm}$ 0.5-0.6, cell count = 7.8×10^8 cells/mL) and incubated at 30°C at 180 rpm for enzyme production. Samples (1 mL) were taken and growth pattern was studied by cell counting using haemocytometer (Superior Marienfeld, Germany). Since the medium contained lipidic substrate which was used as an emulsion, the absorbance (for biomass) was not recorded as it was found to be error prone. Instead the cell counting was done and log number of cells was used for growth and expressed as Log cell/mL. The dry cell weight was also not preferred for the reason that the lipidic substrate interfered at the time of weighing and during the resuspension of pellet for activity determination. Samples taken at regular intervals of time were centrifuged at 13,000 ×g for 10 min at 4°C. Lipase activity was determined initially to determine the location of the enzyme in cells as well as in supernatant using Vorderwülbecke et al. (1992) method [10]. Best concentration of TB giving maximum lipase activity was added to the production medium and supplemented with variable % of methanol (v/v) ranging from 5 to 20% (v/v). The best concentration of methanol and substrate containing media, giving maximum enzyme activity, was further used as basal media (control) for lipase production from identified microorganism.

2.3. Enzyme Assay for Lipase. Lipase activity was determined using Vorderwülbecke et al. (1992) method with slight modification [10]. This method involves measuring the release of *p*-nitrophenol (*p*-NP) by the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) (Sigma, Aldrich USA). 100 μL of enzyme (bacterial cell suspension and supernatant) was added to 0.9 mL of working solution (3 μg of substrate *p*-NPB dissolved in 1 mL of propane 2-ol mixed with solution II containing gum arabic 0.5/450 mL of Tris-HCl and triton X-100 2 g/450 mL) and 2 mL of Tris-HCl buffer of 7.5 pH, 50 mM. The reaction mixture was incubated at 30°C for 10 min and was stopped by placing the reaction tubes in ice. Pale yellow colour appeared due to the enzymatic hydrolysis of *p*-NPB to corresponding *p*-NP which was measured spectrophotometrically by taking absorbance at 410 nm. Amount of *p*-NP released was calculated from standard curve.

One unit of lipase activity was defined as the amount of enzyme required to liberate 1 μg of *p*-NP/mL under standard assay conditions.

Extracellular activity was determined during the initial sets of experiment for lipase production. However, there was almost no activity as compared to the activity with cells (intracellular). Therefore, cells were used to determine the activity in the subsequent experiments. Calculation of intracellular activity was done by suspended wet cells (weighed cells) in buffer (Tris-HCl, pH 7.5, 50 mM). In order to generate a scale for each sample, the activity obtained after calculation was divided by the mg of wet cell weight used in sample preparation (to make it per mg) and expressed as U/mg.

2.4. Enzyme Assay for Protease. Activity for proteases was determined using Keay and Wildi (1970) method [11] with a slight modification. Bacterial cell suspension in phosphate buffer (200 mM, pH 7.0) and supernatant were used to determine the protease activity. 200 μL of enzyme sample was mixed with 500 μL of substrate, that is, casein (1% w/v), and 300 μL of 0.2 M phosphate buffer of pH 7.0 was added. The reaction mixture was incubated at 60°C for 10 min. The reaction was stopped by adding 1 mL of 10% of trichloroacetic acid. The reaction mixture was centrifuged at 13,000 ×g for 10 min. Supernatant was collected and 5 mL of 0.4 M of Na_2CO_3 and 1 mL of Folin reagent were added. The mixture was then incubated at room temperature for 10 min and blue colour obtained was measured spectrophotometrically by taking absorbance at 660 nm. One unit of enzyme activity was defined as the amount of enzyme that librated 1 μg of tyrosine from casein per minute under standard assay conditions. Protease activity was expressed as U/mg for bacterial cells and U/mL for supernatant.

2.5. Identification of Bacterial Isolate. RV68 was selected as the best bacterial isolate on the basis of high enzyme titre during shake flask fermentation in basal medium. The selected isolate was identified using 16S rRNA approach and deposited under International Depository Authority (IDA) IMTECH Chandigarh with an accession number 5618.

2.6. Fermentation Parameters Optimization. The basal media containing TB (8.4 mM), nutrient broth (1.3% w/v), and methanol (5% v/v) was inoculated with 2% (v/v) bacterial seed culture. Methanol (5% v/v) was added to the production medium after sterilization and prior to inoculation. The

bacterial culture was grown at 30°C in a shaking incubator at an agitation speed of 180 rpm and studied for growth and enzyme production. All the experiments were performed in 250 mL flask containing 50 mL medium and samples taken were processed for lipase activity and growth profile study using standards assay protocol.

2.7. Effect of Different Cultural and Physical Parameters

2.7.1. Effect of Carbon and Nitrogen Sources. Effect of different carbon sources on lipase production from *P. stutzeri* MTCC 5618 was studied by supplementing production medium with lipidic sources like Jatropha, mustard, cotton seed, and flaxseed oil (0.5% v/v) and nonlipidic sources like glucose, fructose, sucrose, lactose, maltose, and mannitol (0.5% w/v). The carbon source giving the maximum lipase activity was used in the production medium. The nutrient broth was replaced with different organic and inorganic nitrogen sources (0.5% w/w). The nitrogen sources used were peptone, tryptone, beef extract, yeast extract, urea, ammonium nitrate, ammonium sulphate, sodium nitrate, potassium nitrate, and magnesium nitrate to evaluate their corresponding effect on lipase production. Fermentation was carried out at 30°C and 180 rpm and supplemented with 5% methanol (v/v) prior to inoculation with bacterial seed culture (2% v/v) in 250 mL Erlenmeyer flask. Control containing TB (8.4 mM) and 1.3% nutrient broth (w/v) supplemented with 5% methanol was also run for comparison.

2.7.2. Effect of Metal Ions and Surfactants. Effect of different metal ions like $CaCl_2{\cdot}2H_2O$, $FeCl_2{\cdot}6H_2O$, $CoCI_2$, $MgCl_2{\cdot}6H_2O$, NaCl, $HgCl_2$, and KCI (5 mM) and their subsequent effect on lipase production from *P. stutzeri* MTCC 5618 were also evaluated. The metal ion showing maximum effect was selected for further study in variable molar concentration (5–100 mM (v/v)) to study its effect on the enzyme production. Likewise the effect of different surfactants on lipase production was also studied. The production media were supplemented with different surfactants, namely, SDS (0.05% w/v), triton X-100, tween 20, tween 40, tween 60, and tween 80 (0.05% v/v). Surfactant showed maximum effect on lipase production was further used in variable concentrations (0.025–0.25% (v/v)) to find their influence on lipase production.

2.7.3. Effect of Physical Parameters. The effect of inoculum size on lipase production and growth was studied by inoculating production media with variable percentage of bacterial seed culture ranging from 1 to 6% (v/v), respectively, in previously optimized medium.

Likewise, the effect of pH on lipase production has been studied by varying pH of production media ranging from 5.0 to 12.0. The production media of alkaline pH 10.0, pH 11.0, and pH 12.0 were sterilized at 10 psi for 15 min to protect media from charring. The production media of different pH were supplemented with 2% (v/v) seed culture and incubated at 30°C, 180 rpm. The effect of different temperatures on enzyme production was studied by incubating the production media inoculated with 2% (v/v) bacterial seed culture at different temperatures of 25°C, 30°C, and 37°C, respectively.

Similarly the effect of agitation on solvent tolerant lipase production was also evaluated by incubating the production media containing 2% seed culture at 30°C (optimized temperature in previous experiment) at different agitation speed, that is, static, 140, 180, and 220 rpm. Samples were taken in all the experiments at regular intervals of time and analyzed for enzyme activity using standard assay protocol.

The optimization studies resulted in maximum lipase activity. The optimized media contained jatropha oil (5 g/L), yeast extract (5 g/L), $CaCl_2{\cdot}2H_2O$ (5 mM), tween 40 (0.01% v/v), and physical parameters (temperature (30°C), agitation (180 rpm), pH (9.0), and inoculum (2%)) was used for scaled up in 5 L bioreactor (working volume of 3.5 L) to evaluate growth and enzyme production by RV68.

2.8. Scale-Up of Lipase from P. stutzeri MTCC 5618 in In Situ Bioreactor. Lipase production was scaled up from 250 mL to 5 L laboratory bioreactor with a working capacity of 3.5 L. Bacterial seed culture (2% v/v) was added to the bioreactor under flame and operated under controlled conditions of aeration (0.4 L min^{-1}), agitation (180 rpm), pH 7.0 (basal medium containing TB 8.4 mM and nutrient broth 1.3 w/v) and 9.0 (optimized media), and temperature (30°C). Samples were withdrawn at regular intervals of time under flame to avoid contamination and analyzed for lipase activity and growth profile. Protease activity was also determined during scale-up study to find its possible effect on lipase production.

3. Results and Discussion

3.1. Identification of Bacterial Isolate. The bacterial isolate, RV68, was selected on the basis of high enzyme titre during shake flask fermentation and identified using 16S rRNA approach. BLAST (Basic Local Alignment Search Tool) was performed for matching from data base where strain showed 100% homology with *Pseudomonas stutzeri* (Figure 1).

In order to find evolutionary relationship, phylogenetic tree was constructed using MEGA5 software for *P. stutzeri* and inferred using Neighbor-Joining method [12, 13].

The optimal tree with the sum of branch length was 9.70 as calculated automatically by MEGA5 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches [14]. The evolutionary distances were computed using the Maximum Composite Likelihood method [15] and are in the units of the number of base substitutions per site. The use of traditional identification of bacteria which is based upon the phenotypic characteristics is currently not recognised as accurate method for identification with respect to genotypic methods. 16S rRNA technique for gene sequence analysis on the other hand can better identify poorly described, rarely isolated, or phenotypically aberrant strains, which can be routinely used for identification of number of microbial isolates which further can be used to study novel application [16].

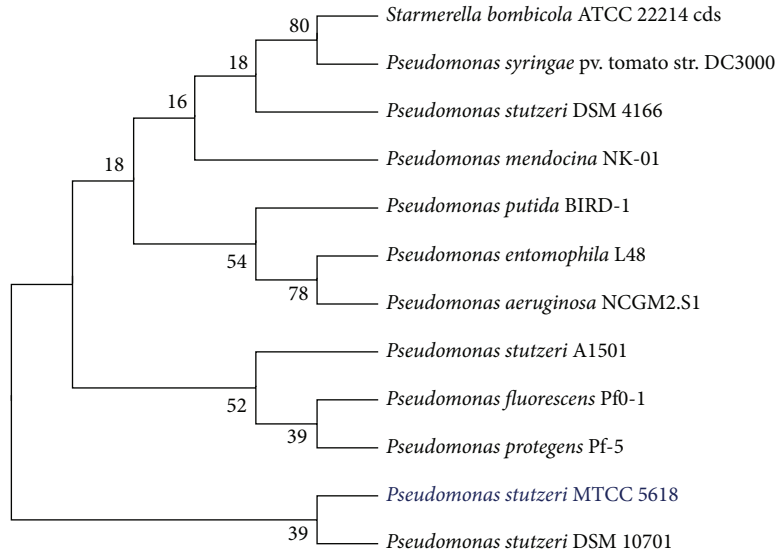

Figure 1: Evolutionary relationship for *P. stutzeri* MTCC 5618.

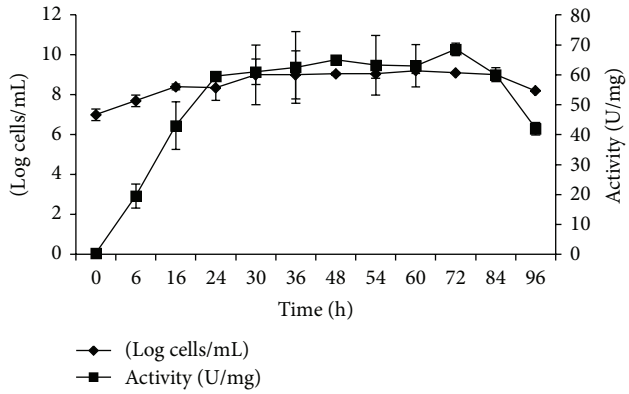

Figure 2: Growth and lipase production profile for *P. stutzeri* MTCC 5618 in basal medium containing TB (8.4 mM) as carbon source.

3.2. *Effect of Different Physicochemical Factors on Solvent Tolerant Lipase Production from Pseudomonas stutzeri MTCC 5618*

3.2.1. Effect of Carbon Sources. Initially shake flasks time course study in basal media containing TB (8.4 mM) was found to be giving the maximum lipase activity of 68 U/mg (wcw) after 72 h of fermentation followed by decline in lipase activity (Figure 2). Lipase production commences after 6 h and increases as the biomass increases. This was taken as basal media and used as control in subsequent studies. Lipase production in the present study was found to be growth associated which increased with increase of biomass and attained optima at maximum growth followed by decline phase.

Most of the lipases studied so for are reported to be inducible and require some carbon source for their synthesis and expression. These carbon sources may be some lipidic or nonlipidic substrates which trigger the associated genes responsible for the expression of lipase proteins [17].

In the present study, different substrates like lipidic (0.5% v/v) and nonlipidic (0.5% w/v) ones were used to enhance lipase production from *P. stutzeri* MTCC 5618. *P. stutzeri* MTCC 5618 showed maximum biomass formation of 8.8 Log cells/mL and enzyme production of 68 U/mg in jatropha oil containing production medium at 72 h of fermentation. Lipase activity was equivalent to that in basal media containing TB as carbon source. Lipase activity starts after 18 h of fermentation and increased with increased biomass till 72 h followed by decrease in activity and biomass, respectively (Figure 3(a)). Lipase production and biomass formation increased in flaxseed oil containing medium till 72 h giving maximum activity of 67 U/mg and 8.4 Log cells/mL, followed by decline in cell number and activity. However the growth maxima and activity maxima in mustard oil containing production medium were at 48 h giving 8.7 Log cells/mL and 61.66 U/mg of activity followed by a slow decrease. Presence of cotton seed oil in production medium does not influence the lipase production, although the cell number in medium was quite close to that in control medium. Among carbohydrates, only maltose exhibited significant effect on enzyme production which increases till 72 h giving 45% of activity and 8.2 Log cells/mL followed by a slow decline phase. *P. stutzeri* MTCC 5618 showed growth in different carbohydrates containing medium, but the production was significantly less, which revealed that carbohydrates were not effective to enhance lipase production from *P. stutzeri* MTCC 5618 (Figure 3(b)).

The loss of activity in the presence of different carbohydrate might be due to the catabolite repression [18]. In contrast to the present finding, cotton seed and mustard oil were found to be the best carbon source for lipase production from *B. licheniformis* MTCC 10498 and *Pseudomonas* sp. after 72 h of fermentation [8, 19]. Some studies highlight the role of

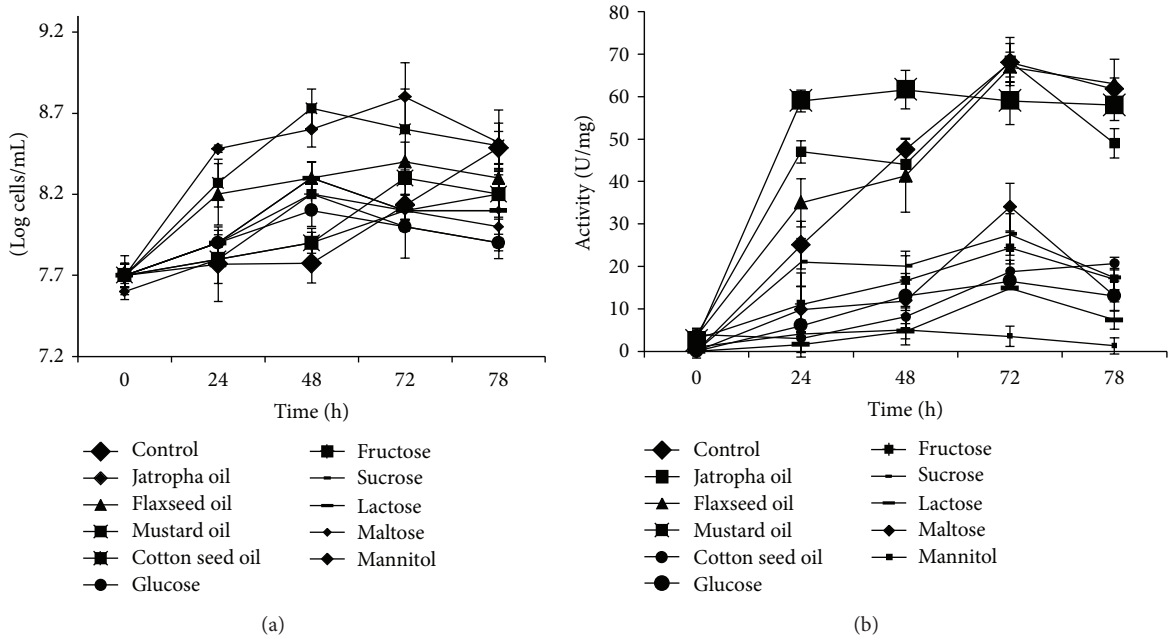

Figure 3: (a) Growth profile of *P. stutzeri* MTCC 5618 in the presence of different lipidic and nonlipidic carbon sources. (b) Lipase production by *P. stutzeri* MTCC 5618 in the presence of different lipidic and nonlipidic carbon sources.

carbohydrates like starch as the best carbon source for lipase production [2]. Mannitol (0.2% w/v) was reported as the best carbon source for lipase production from *B. megaterium* AKG-1 [20].

3.2.2. Effect of Different Nitrogen Sources. In the present investigation yeast extract was found to be the best nitrogen source for maximum lipase production, increasing activity 1.57-fold with respect to control. Cell number and enzyme production in yeast extract (0.5% w/v) containing medium increase with increase in fermentation time till 72 h giving 8.8 Log cells/mL and 96 U/mg of activity followed by steep decline for both. Other organic nitrogen sources like beef extract and tryptone yield lipase activity of 0.55-fold and 0.44-fold with respect to control after 72 h of fermentation. However maximum biomass of 8.2 Log cells/mL in tryptone containing medium was observed at 72 h whereas maximum lipase activity of 55 U/mg was observed at 48 h. Beef extract containing medium showed maximum cell number of 7.5 Log cells/mL and 45 U/mg activity maxima at 48 h of fermentation followed by decline phase. In contrast to other organic nitrogen sources, peptone does not influence the lipase production (10 U/mg) although the biomass was approximately high as in case of tryptone containing medium. Among different inorganic nitrogen sources, both biomass formation and lipase activity were significantly inhibited (Figures 4(a) and 4(b)).

However in comparison to peptone, inorganic nitrogen sources like ammonium sulfate, sodium nitrate, potassium nitrate, and magnesium showed high activity giving 12.5 U/mg, 18 U/mg, 26 U/mg, and 16 U/mg after 48 h of fermentation. Our results are in concordance with the earlier proposed studies that the yeast extract is the best nitrogen source for lipase production by *B. licheniformis* MTCC 10498, whereas other nitrogen sources like peptone were reported as the best source for enzyme production by *Pseudomonas* sp. [8, 19].

3.2.3. Effect of Different Inoculum Size. In the present study, among varied % of inoculums, 2% (v/v) seed culture showed maximum lipase activity giving 1.06-fold after 72 h with respect to control. Increased inoculum percentage from 3 to 6% (v/v) leads to decrease in enzyme activity, whereas 1% (v/v) inoculum addition to production medium yields 83.33% lipase activity with respect to control after 72 h of fermentation. Production medium inoculated with 3–6% (v/v) seed culture showed growth associated lipase production with maximum biomass of 8.6 Log cells/mL, 8.4 Log cells/mL, 8.6 Log cells/mL, and 8.56 Log cells/mL and lipase activity of 37 U/mg, 48 U/mg, 45 U/mg, and 36 U/mg, respectively, at 48 h of fermentation followed by slow decline for both. However the maximum biomass and lipase activity at 78 h giving 8.5 Log cells/mL and 55 U/mg of lipase activity were observed in 1% (v/v) added bacterial seed culture, followed by a small decline in activity and cell number (Figures 5(a) and 5(b)).

Addition of optimum number of culture cells in production media plays an important role in enzyme production. The more the number of cells in production media the more the competition for substrate and vice versa. The availability of nutrients and oxygen level was sufficient to support bacterial growth and lipase production is more in low inoculum added medium, whereas with the increased percentage of inoculum size in production medium, the availability of

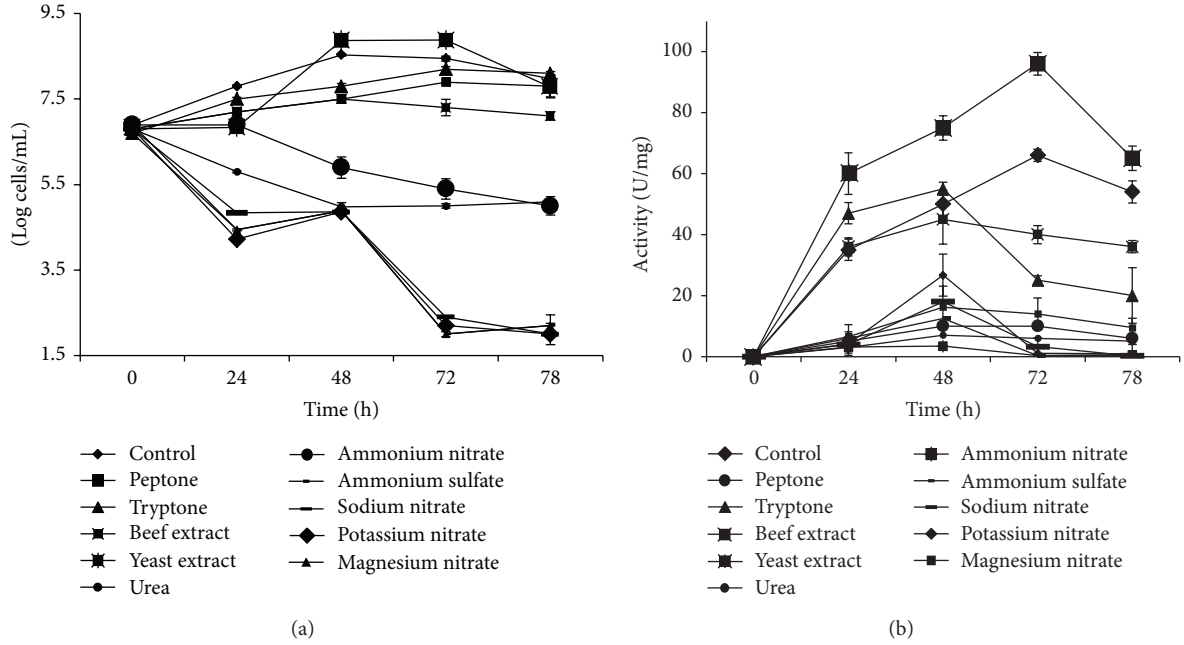

Figure 4: (a) Growth profile of *P. stutzeri* MTCC 5618 in the presence of different organic and inorganic nitrogen sources. (b) Lipase production by *P. stutzeri* MTCC 5618 in the presence of different organic and inorganic nitrogen sources.

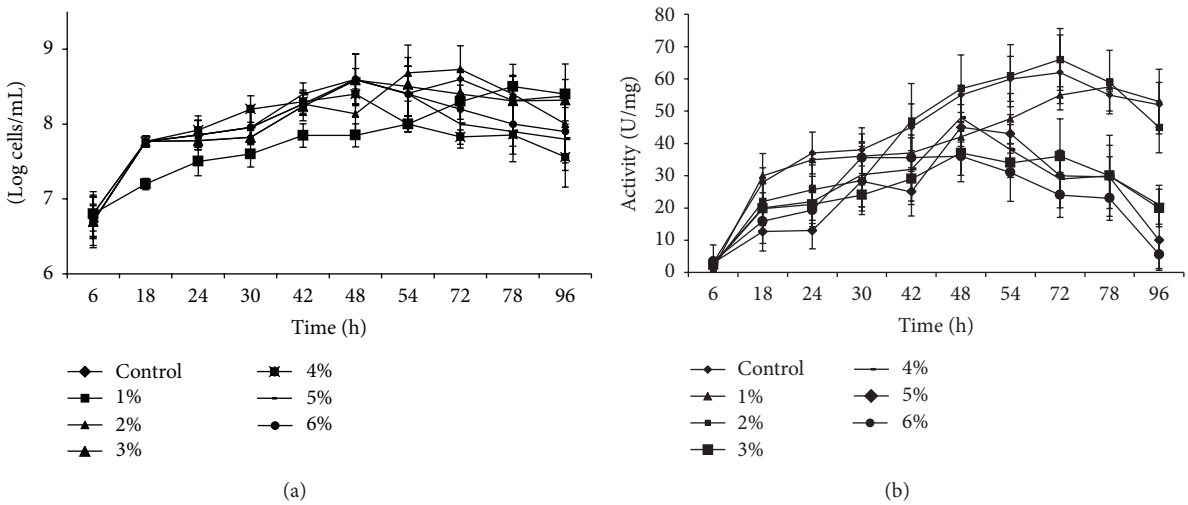

Figure 5: (a) Effect of different inoculum size on growth pattern of *P. stutzeri* MTCC 5618. (b) Effect of different inoculum size on lipase production by *P. stutzeri* MTCC 5618.

nutrient and oxygen might be a limiting factor. However, some finding reveals that high percentage of inoculum also leads to high enzyme production and vice versa [21, 22]. For example, Chauhan and Garlapati (2013) reported maximum lipase activity of 0.349 U/mL after 48 h of fermentation by *Staphylococcus arlettae* JPBW-1 when the production medium was inoculated with 10% (v/v) of seed culture [23].

3.2.4. Effect of pH. The effect of pH on growth and lipase production from *P. stutzeri* MTCC 5618 was studied by varying the pH of media from 5 to 12. *P. stutzeri* MTCC 5618 showed growth and lipase production in the acidic as well as alkaline range. Lipase production was found to be growth dependent and increased with the increase in biomass and retarded once the cell number decreased in medium. Production medium of pH 9.0 found to giving maximum enzyme activity of 78 U/mg with 1.18-fold with respect to control. The biomass increased with time till 72 h of fermentation giving 8.7 Log cells/mL, followed by decline in activity and biomass. Lipase activity was maximum in pH 10 media (117 U/mg) after 42 h of fermentation with maximum biomass of 7.85 Log cells/mL followed by a gradual decrease in both cell number and lipase activity. In acidic conditions

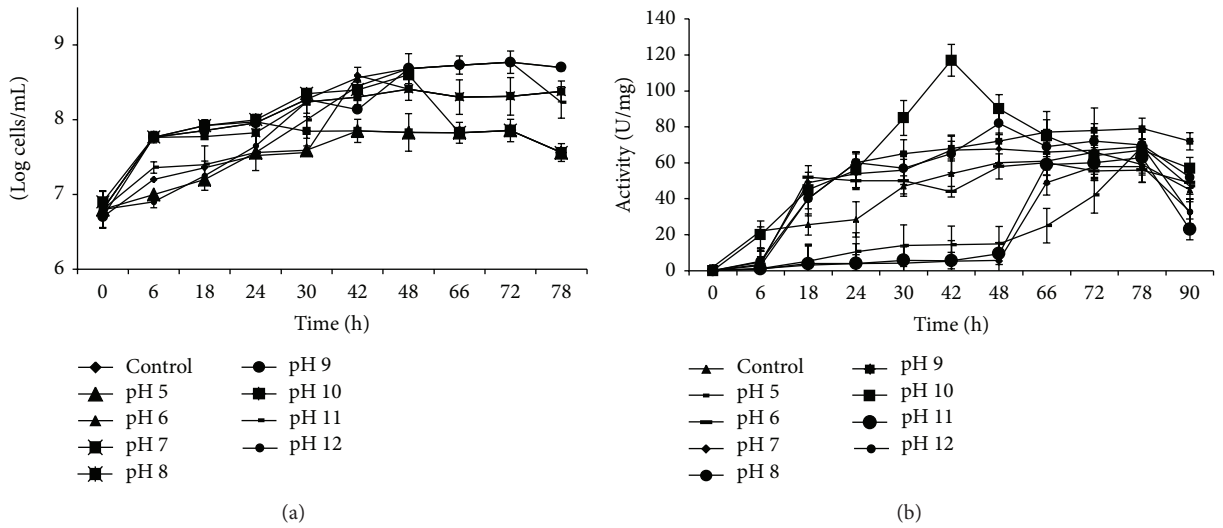

Figure 6: (a) Effect of different pH on growth of *P. stutzeri* MTCC 5618. (b) Effect of pH on lipase production by *P. stutzeri* MTCC 5618.

(pH 5.0), enzyme activity was 69 U/mg after 78 h of fermentation with maximum biomass of 7.85 Log cells/mL followed by decline phase. Similar trend for biomass and activity from *P. stutzeri* MTCC 5618 was found at other acidic and alkaline ranges (Figures 6(a) and 6(b)).

In contrast to present results, *Fusarium globulosum* showed maximum lipase production in neutral pH conditions after 96 h of fermentation followed by a decline in activity [24]. pH plays a significant role in enzyme stability as it maintains the necessary three-dimensional structure required for biological activity of enzyme. Since enzymes are proteins in nature and the state of pH has great impact on the ionization state of amino acids which in turn form the primary and secondary structure of enzyme and controlling the overall activity, a change in pH directly affects the structure, stability, and activity of biocatalyst [7]. Fungal lipases are reported to produce high enzyme titre under acidic conditions whereas bacterial isolates were reported to produce lipase in alkaline conditions [25, 26]. Sharma et al. (2012) reported optimum pH 7.5 for maximum lipase production giving 0.389 U/mL of activity by *B. licheniformis* MTCC 10498 after 72 h of fermentation, whereas in alkaline conditions (pH 9.0) enzyme activity decreases and yields 84% of initial activity [8].

3.2.5. Effect of Temperature. Effect of different incubation temperatures on lipase production in previously optimized media was determined at 25°C, 30°C, and 37°C at 180 rpm. Maximum activity was observed at 30°C with 1.34-fold with respect to control containing TB (8.4 mM as carbon source). However, at 37°C activity was 1.19-fold after 72 h of fermentation with respect to control. At 25°C growth pattern for bacterial strain was quite uniform and increases with fermentation time and showed growth maxima at 72 h giving 7.85 Log cells/mL followed by a drastic decline in growth till 78 h giving 7.5 Log cells/mL. Lipase activity increased along with increased biomass and showed uniform activity from 48 h to 72 h, giving maximum activity of 51 U/mg at 72 h of fermentation followed by drastic decrease at 78 h (21 U/mg). When incubated at 30°C, *P. stutzeri* MTCC 5618 showed uniform growth pattern and lipase production attaining maxima at 72 h giving 8.76 Log cells/mL and 86 U/mg followed by decrease for both. At 37°C maximum for growth was attainted at 48 h giving 8.59 Log cells/mL, whereas activity maximum was found at 72 h (70 U/mg). After attaining the maximum biomass at 48 h, decline phase was observed (Figures 7(a) and 7(b)).

Some study reveals that higher temperature is suitable factor for maximum biomass formation, whereas 30°C was reported suitable for enzyme production from fungal culture [26]. In contrast to the present findings, Rifaat et al. (2010) reported maximum lipase production from *F. oxysporum* at 28°C. Activity decreases and diminishes at the incubation temperature of 45°C. These results showed that lipase production is species specific and has its own temperature optima which favoured maximum enzyme production [27].

3.2.6. Effect of Metal Ions. In the present investigation, addition of Ca^{2+} (5 mM) ion to the production medium increased lipase activity by 1.47-fold with respect to control. In production medium containing Ca^{2+} and Mg^{2+} ions, the biomass starts increasing till 72 h giving 8.6 Log cells/mL and 8.64 Log cells/mL followed by slow decline in cell number. Similar trend was observed when analyzed for lipase production, which increased with increased biomass till 72 h giving maximum activity of 162 U/mg and 96 U/mg followed by decline. In the Na^{+} and K^{+} metal ions containing production media, increase in cell number was observed till 48 h giving 8.6 Log cells/mL followed by decline. However, maximum lipase activity of 96 U/mg and 72 U/mg was observed during initial hrs of fermentation, that is, after 18 h, where the cell number was 7.7 Log cells/mL in both production media, followed by a gradual decline till 48 h and drastic decline.

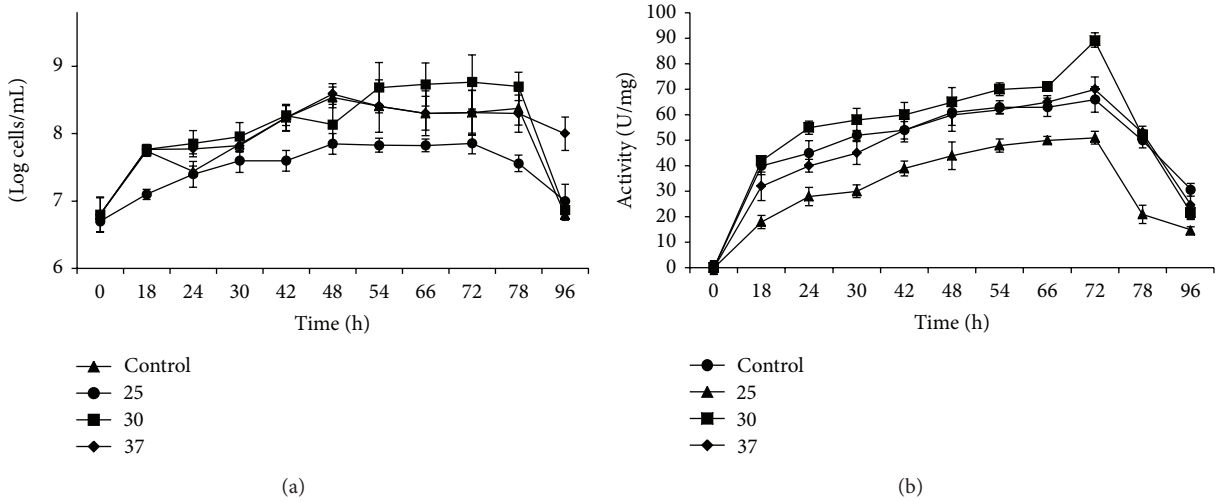

Figure 7: (a) Effect of different incubation temperatures on growth of *P. stutzeri* MTCC 5618. (b) Effect of different incubation temperatures on lipase production by *P. stutzeri* MTCC 5618.

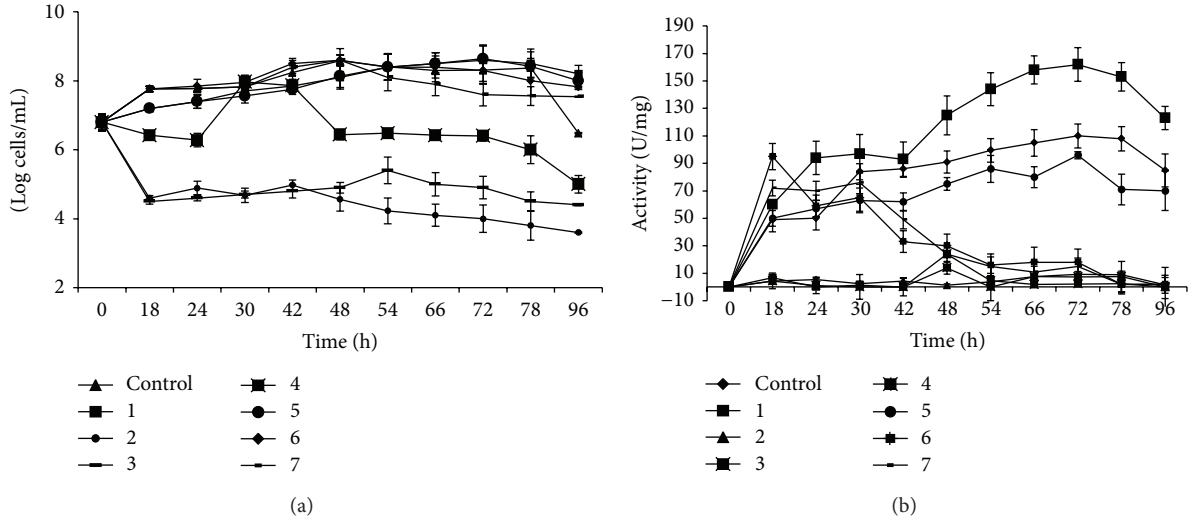

Figure 8: (a) Effect of metal ions (5 mM) on growth of *P. stutzeri* MTCC 5618. Numeric number represents 1: Ca^{2+}; 2: Co^{2+}; 3: Hg^{2+}; 4: Fe^{2+}; 5: Mg^{2+}; 6: Na^{+}; 7: K^{+}. (b) Effect of metal ions (5 mM) on lipase production by *P. stutzeri* MTCC 5618. Numeric number represents 1: Ca^{2+}; 2: Co^{2+}; 3: Hg^{2+}; 4: Fe^{2+}; 5: Mg^{2+}; 6: Na^{+}; 7: K^{+}.

The maximum activity was found to be 1.9-fold and 1.46-fold higher in comparison to control, whereas Ca^{2+} ions showed 1.22-fold activity after 18 h of fermentation. However, the addition of Hg^{2+}, Fe^{2+}, and Co^{2+} metal ions inhibited lipase production significantly giving 24 U/mg, 14 U/mg, and 9.24 U/mg lipase activity after 48 h and 72 h, respectively (Figures 8(a) and 8(b)).

Among different concentrations of Ca^{2+} (5–100 mM v/v), maximum effect on growth and lipase production was observed with 5 mM concentration, whereas at 100 mM concentration, activity was significantly inhibited (Figure 9(a)). Biomass and lipase production start increasing with the fermentation and reached the maxima at 72 h giving 8.6 Log cells/mL and 180 U/mg followed by decline. Similar trend was observed in 25, 50, 75, and 100 mM concentrations, respectively. In all the concentrations of Ca^{2+} ions both biomass and lipase activity optima were attained at 72 h of fermentation followed by decline. However, with the increase in Ca^{2+} concentration, corresponding decrease in cell number and lipase activity was observed. This indicated that metal ions are generally required in low concentration to activate or induce maximum enzyme production (Figures 9(a) and 9(b)).

For lipase production, metal ions are known to play a major role. Presence of different metal ions in production media has different promotory or inhibitory influence on enzyme production [26]. The promotory effect of Ca^{2+} ions was due to formation of complex with released fatty acids

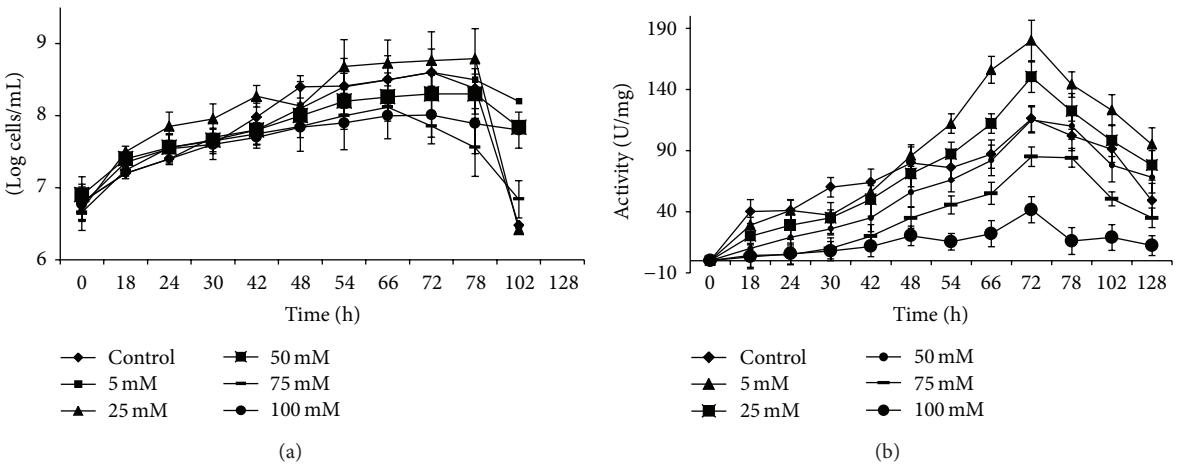

Figure 9: (a) Effect of variable concentration of Ca^{2+} on growth of *P. stutzeri* MTCC 5618. (b) Effect of Ca^{2+} ion on lipase production by *P. stutzeri* MTCC 5618.

from back bone of lipid substrate during hydrolysis at interface which changed the behaviour and solubility of enzyme towards oil-water interface [28]. Many reports confirm the presence of metal ions like Ca^{2+}, Mg^{2+}, K^{+}, and Hg^{2+} for their role as activator inhibitor in lipase production from bacterial and fungal sources [6, 28]. Açikel et al. (2011) reported the presence of metal ions Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} (0.5 g/L) increased the lipase activity from *R. delemar* by 1.91-, 1.85-, 1.83-, 1.76-fold, respectively. However, the maximum biomass (4.44 g dcw/L) was observed in medium containing Ca^{2+} ion, whereas Fe^{2+} and Hg^{2+} are known to inhibit lipase activity [29].

3.2.7. Effect of Surfactants. In the present study, among different surfactants, maximum lipase production was induced by tween 40 (252 U/mg), followed by tween 60, tween 80, and tween 20 giving 171 U/mg, 142 U/mg, and 160 U/mg activity, respectively, after 72 h of fermentation with respect to control. However, SDS and triton X-100 were found to inhibit the activity by 0.77-fold (23 U/mg) and 0.58-fold (42 U/mg), respectively.

In the time course fermentation, growth and lipase production from *P. stutzeri* MTCC 5618 were evaluated at different intervals of time. Biomass production and lipase activity in the presence of tween 20, tween 40, and tween 60 increased along with fermentation time till 72 h followed by decline in cell number. However, maximum production and cell number were found in the presence of tween 40 containing production medium giving 8.58 Log cells/mL and 252 U/mg of activity. In the presence of tween 60, biomass and lipase activity followed the same trend, showing maximum cell number and lipase activity of 8.3 Log cells/mL and 171 U/mg after 72 h followed by decline phase. Presence of tween 80 in production media showed maximum biomass and activity at 42 h giving maximum cell number of 7.8 Log cells/mL and 150 U/mg of activity followed by slow decline till 78 h. In the presence of anionic detergent like SDS, biomass generation was significantly inhibited with increased fermentation time; however the lipase activity increased till 54 h giving 43 U/mg of activity and decreased drastically till 78 h (20 U/mg). Similar trend was observed for biomass generation in the presence of nonionic detergent triton X-100. Biomass was decreased along with increase in fermentation time but the lipase production was higher than observed for SDS showing increase in activity till 48 h (66 U/mg) followed by a gradual decline (Figures 10(a) and 10(b)).

Among variable concentrations of tween 40, maximum biomass formation and lipase activity were observed in production medium containing 0.10% (v/v) tween 40. Cell number and lipase production increased with fermentation time and showed maxima at 72 h giving 8.7 Log cells/mL and 221 U/mg, followed by gradual decrease for both. The increase in lipase production in 0.10% (v/v) tween 40 containing production medium was 1.9-fold when compared with control. Similar trend of growth and lipase activity was observed in the presence of 0.025% (v/v) and 0.05% (v/v) concentration of tween 40 giving 8.4 Log cells/mL and 8.6 Log cells/mL of cell number and 182 U/mg and 196 U/mg lipase activity after 72 h of fermentation. However the cell number in production medium was less when compared to 0.10% tween 40 containing production medium. This showed that the 0.10% (v/v) tween 40 enhanced the growth of *P. stutzeri* MTCC 5618 significantly.

Higher concentration of tween 40 (0.15, 0.20, and 0.25% (v/v)) decreases both biomass and lipase activity. *P. stutzeri* MTCC 5618 showed less growth and eventually low production of lipase at higher concentrations of tween 40. In the presence of 0.150% (v/v) tween 40, maximum growth and lipase activity were attained at 42 h giving 7.8 Log cells/mL and 44 U/mg of enzyme activity followed by a decrease. However in 0.20% (v/v) and 0.25% (v/v) concentration, maximum cell number and lipase activity were achieved after 30 h of fermentation. Maximum cell number of 7.9 Log cells/mL and 49 U/mg of lipase activity were observed in 0.2% (v/v) tween 40, whereas in 0.25% (v/v) tween 40 maximum cell number of 7.6 Log cells/mL and 27 U/mg of activity were achieved after

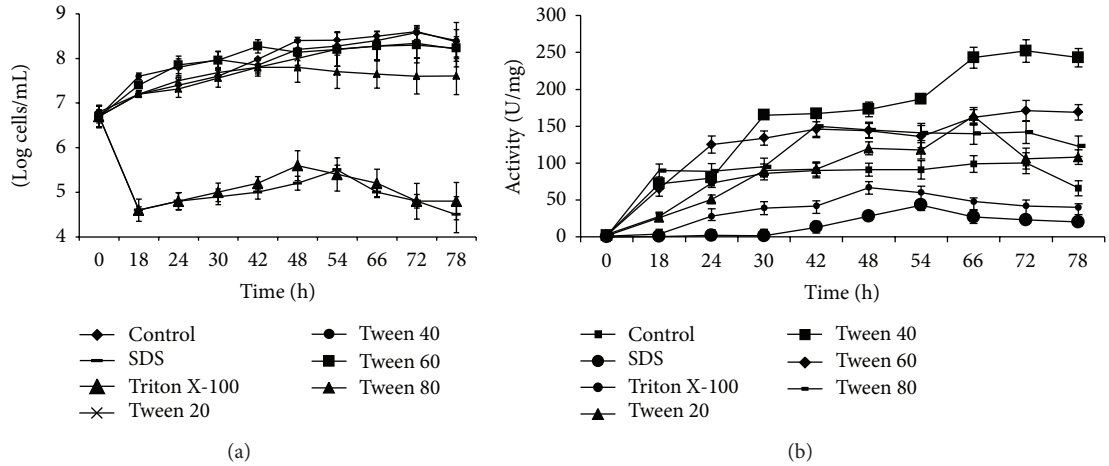

Figure 10: (a) Effect of different surfactants on growth of *P. stutzeri* MTCC 5618. (b) Effect of different surfactants on lipase production by *P. stutzeri* MTCC 5618.

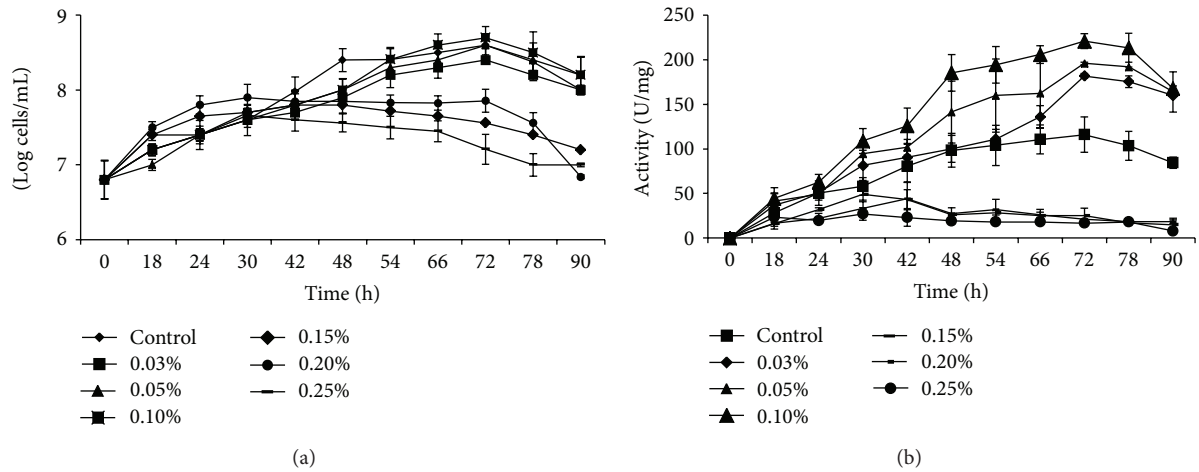

Figure 11: (a) Effect of variable concentrations of tween 40 on growth of *P. stutzeri* MTCC 5618. (b) Effect of variable concentrations of tween 40 on lipase production by *P. stutzeri* MTCC 5618.

30 h followed by gradually decrease in both production media (Figures 11(a) and 11(b)).

Emulsifying agents are known to enhance the substrate uptake in the medium by reducing the surface tension at the interface site and thus eventually lead to the production of enzyme. The results obtained in the present study were in contrast with those reported for lipase production by *C. cylindracea* where the presence of tween 80 in 0.65% (v/v) in optimized production medium enhanced the enzyme production by 5.19-fold higher than the unoptimized media [22].

3.2.8. Effect of Agitation. In the present exercise, maximum enzyme production was found when fermentation was studied at 180 rpm, giving 1.62-fold increase with respect to control after 72 h of fermentation. The cell number and lipase activity increased gradually with increased biomass and reached to the maxima at 72 h of fermentation giving 8.7 Log cells/mL and 190 U/mg, followed by decline of both. However, at low agitation of 140 rpm, biomass generation was less when compared to biomass formation at 180 rpm. The cell number at 140 rpm increased till 48 h giving maxima of 7.4 Log cells/mL followed by gradual decrease. Lipase activity also showed maxima at 48 h giving 64 U/mg followed by steady decrease along with declined cell number. In contrast to this, at 220 rpm, both cell number and lipase activity were found to increase till 66 h of fermentation giving 8.2 Log cells/mL and 68 U/mg activity followed by decline phase. *P. stutzeri* MTCC 5618 was also found to yield lipase activity when the production flask inoculated with culture was incubated at 30°C on static condition. Both cell number and corresponding lipase activity were found to achieve maxima during early phase of fermentation at 30 h giving 6.84 Log cells/mL and 38 U/mg of lipase activity, followed by slow decline in both. However, the biomass at static condition was quite less when compared to control. This indicates that the agitation contributes to both bacterial growth and enzyme production (Figures 12(a) and 12(b)).

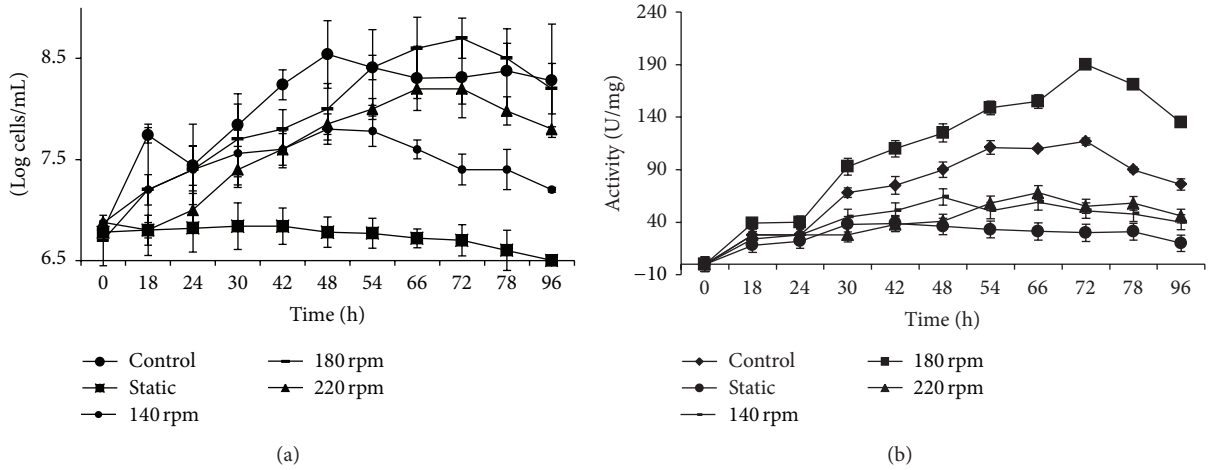

Figure 12: (a) Effect of agitation on lipase production by *P. stutzeri* MTCC 5618. (b) Effect of different agitation on lipase production by *P. stutzeri* MTCC 5618.

In contrast to the present study, Kumar et al. (2013) reported maximum production of enzyme from *Bacillus* sp. of 48 IU/mL at 700 rpm [30]. The variation in biomass and lipase activity at different agitation rate in the present study might be due to beneficial or pernicious influence on cell morphology, cell wall, and cell growth due to different rate of substrate utilization which ultimately influenced the product yield [31]. Lipase production by *Pseudomonas* sp. strain S5 reported for maximum activity when incubated in static conditions [30]. Primary role of agitation is to ensure proper mixing of oxygen in fermentation broth that ultimately becomes available for bacterial culture for growth in the form of dissolved oxygen [32]. Low agitation influences the OTR in fermentation broth because proper mixing of air with media does not happen, whereas at high agitation, mechanical shearing may happen which may ultimately influence the biomass formation and enzyme production. However, in shake flask experiments, it is very difficult to correlate the effect of OTR and agitation speed on biomass generation and enzyme production [31].

3.2.9. Protease Activity from P. stutzeri MTCC 5618 in Optimized Media. Coproduction of proteases along with lipase also was accessed to check its effect on lipase production during shake flask fermentation in optimized media. Maximum proteases activity was observed after 54 h of fermentation for cells and supernatant (0.65 U/mg and 0.855 U/mL) followed by a decline in activity (Figure 13). In contrast to this, the intracellular lipase activity was 149 U/mg after 54 h of fermentation and achieved maximum activity after 72 h of fermentation. Production of proteases was accessed to find its effect on simultaneous production of lipase since proteases are known to degrade other proteins. However the lipase production remains unaffected since the production of proteases activity is very small in comparison to lipase production during fermentation.

3.3. Scale-Up of Lipase Production from P. stutzeri MTCC 5618. Scale-up for lipase production from *P. stutzeri* MTCC 5618

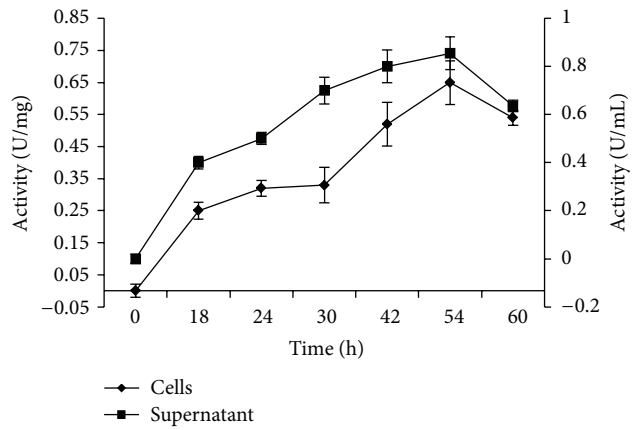

Figure 13: Protease activity of *P. stutzeri* MTCC 5618 in shake flask in optimized media (pH 9.0).

was carried out in *in situ* bioreactor (5 L) in controlled conditions of temperature (30°C), agitation (180 rpm), and aeration (0.4 LPM). In time course fermentation during scale-up in optimized medium, the activity for lipase starts from 18 h of fermentation and attained growth (7.8 Log cells/mL) and activity maxima at 48 h (99 U/mg) followed by a decline phase (Figure 14). The decline in lipase activity can be correlated with the decline in cell number which showed that lipase production was growth associated.

In order to evaluate the effect of protease activity on lipase production during the fermentation, protease activity was also determined (Figure 15). Protease activity increased with the fermentation time when analyzed for intracellular production even after 48 h of fermentation giving 1.6 U/mg activity after 60 h of fermentation. In supernatant, protease activity was maximum after 18 h giving 1.6 U/mL of activity followed by decline as the fermentation time increases. We interpret from this study that lipase production in the present study by *P. stutzeri* MTCC 5618 remained unaffected when compared to protease production.

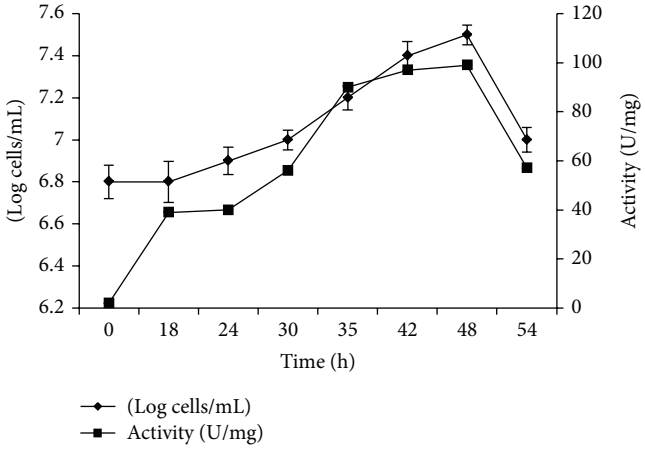

Figure 14: Growth & lipase production by *P. stutzeri* MTCC 5618 in 5 L *in situ* bioreactor in optimized media (pH 9.0).

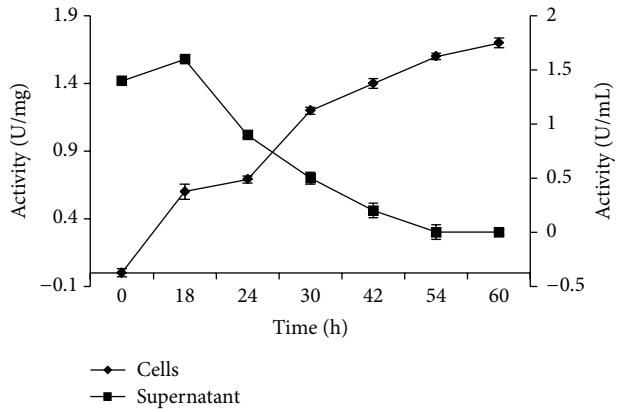

Figure 15: Protease activity profile for *P. stutzeri* MTCC 5618 during scale-up study in optimized media (pH 9.0).

Other bacterial strain like *P. fluorescens* NS2W was reported to yield maximum lipase activity of 68.7 U/mL after 48 h at pH of 7.2 and 30°C in 1 L bioreactor [33]. *P. fluorescens* RM4 reported for maximum lipase activity during late logarithmic phase, followed by a decline with the onset of stationary phase [34].

In the present study, lipase production in basal medium containing TB (8.4 mM) and NB (1.3% w/v) supplemented with 5% methanol was also studied. In the time course fermentation lipase activity and biomass increased with respect to time till 48 h giving maximum activity of 41 U/mg and 8.4 Log cells/mL, followed by decrease in activity and biomass (Figure 16). The lipase production observed in optimized media during scale-up was 48% less than observed in shake flask. Although the biomass generation in optimized media during fermentation was less when compared to basal media during scale-up study, the increase in activity was 2.4-fold at 48 h of fermentation.

Sangeetha et al. (2010) reported concomitant production of both lipase and proteases by *B. licheniformis* VSG1. TB was used as a potential substrate for lipase production whereas

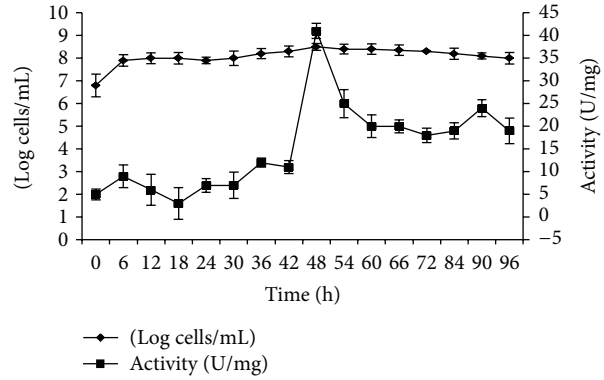

Figure 16: Growth and enzyme production by *P. stutzeri* MTCC 5618 in *in situ* bioreactor (5 L) in basal media.

yeast extract was found to be the best nitrogen source for protease production. Addition of tween 80 in the production media showed enhanced production for lipase. Both lipase and protease showed maximum activity after 72 h of fermentation [35]. These results support our finding where the protease production in shake flask as well as during scale-up in optimized media does not influence the lipase production from *P. stutzeri* MTCC 5618. This further can enhance the application potential of lipase for detergent industry because of its tolerance to protease enzyme degradation.

4. Conclusion

Lipase mediated biocatalysis in organic solvents with its associated advantages has received widespread attention. Therefore maximum production of enzyme with high activity is still an important filed of investigation. Maximum production of industrially important enzymes like lipase through media engineering is still holding its place. The present investigation, enhanced lipase production to 1.6-fold, was achieved through media engineering. Yeast extract, Ca^{2+}, and tween 40 enhanced the lipase production from *P. stutzeri* MTCC 5618 significantly. Similarly best environment factors like inoculums size, temperature, agitation, and pH also showed positive effect on lipase production. Although scale-up study using optimized medium showed decrease in activity, the fermentation time reduced to 48 h as compared to shake flask which compensated the loss in activity. Reduction in fermentation time during scale-up made it more economical in terms of time and energy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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