JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

UTILIZATION OF WHEY AS A CHEAP SUBSTRATE FOR THE OPTIMIZATION OF LIPASE PRODUCTION BY *Bacillus subtilis* B10 ISOLATED FROM DAIRY INDUSTRY

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doi: 10.15414/jmbfs.2017.7.2.193-198

ARTICLE INFO

Received 25. 5. 2017
Revised 13. 6. 2017
Accepted 26. 8. 2017
Published 1. 10. 2017

Regular article

OPEN ACCESS

ABSTRACT

Whey, a by-product of the dairy industry, acts as a prime source of environmental pollution due to its high organic load. Its utilization as a substrate for lipase production is an attractive option in facing the challenge associated with whey disposal. However, there are only very few studies that have reported the production of lipase using whey. In this study, 43 bacterial strains were isolated from dairy industry soil sample, of which, 26 were found to produce lipase. Since whey contains lactose as the sole carbon source, the ability of the isolates to utilize this sugar was tested and two of the isolates B3 and B10 were positive for growth in phenol red lactose broth. When they were cultured by submerged fermentation and their lipase activities quantified, strain B10 displayed 0.79U/ml of activity. In order to improve the production of lipase, one-factor-at-a-time method was used to study the impacts of oil inducers, nitrogen sources, mineral salts and whey concentration on the process. Statistical optimization was performed using the Box-Behnken design of Response Surface Methodology (RSM). From this design, 75% whey, 4% (v/v) sunflower oil, 0.625% (w/v) beef extract and 0.2% (w/v) $CaCl_2$ were inferred to be the optimal conditions that resulted in a maximum lipase activity of 0.954U/ml. Analysis of variance indicated statistical significance of the model. Lipase being an industrially sought-after enzyme owing to its unique properties, this cost-effective route to its production from a waste substrate holds paramount environmental and economic significance.

Keywords: Box-Behnken design; lactose; lipase; one-factor-at-a-time; response surface methodology

INTRODUCTION

Whey, resulting from the processes of cheese making and casein manufacturing, is one of the by-products of dairy industry. It possesses only 6-7% of dry matter, which is far lower when compared to that of milk and hence commonly considered as a waste product. Its generation in large amounts represents a significant environmental problem as it exerts a high Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). However, it contains much of the nutrients present in milk including lactose, lipids, functional proteins, peptides, minerals and vitamins. Lactose makes up a high proportion (47.5%) of the total whey solids and largely contributes to it being considered as one of the most polluting by-product streams. Whey offers immense potential as a source of value added compounds. Therefore, the industry should view the surplus availability of whey as a promising resource and not only as a problem of waste management. Various means of whey disposal and utilization have been described in many reviews. In this study, it has been utilized as a substrate for the production of an industrially important enzyme, lipase.

Lipase is a triacylglycerol acylhydrolase (EC 3.1.1.3) that catalyses the hydrolysis of triglycerides into glycerol and free fatty acids. Unlike esterases (EC 3.1.1.1 carboxylic ester hydrolases), true lipases are able to hydrolyse esters of long-chain fatty acids. They exhibit enantioselective properties. Lipases are produced ubiquitously and possess considerable physiological significance and a wide range of industrial applications. Other than triglycerides, the enzyme possesses active site for other substrates such as aliphatic, alicyclic, bicyclic and aromatic esters and even esters based on organometallic sandwich compounds. The catalytic triad, Serine105-Histidine224-Aspartate187, is present at the enzyme's active site **(Uppenberg *et al.*, 1994a,b)**.

The natural substrates of lipases are practically insoluble in water and the reaction is catalysed only at the water-lipid interface. Besides being lipolytic, lipases also possess esterolytic activity and thus have a very diverse substrate range **(Jaeger *et al.*, 1999)**. The ability of lipase to undergo reverse reaction and synthesize triacylglyceride from glycerol and free fatty acids, discloses that it can catalyse a wide range of reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis **(Joseph *et al.*, 2008)**. It thus displays enzyme promiscuity, an ability to catalyse alternative reactions that differ from the natural physiological reaction.

Microbial lipases are mostly extracellular and their production is greatly influenced by medium composition, besides physicochemical factors such as temperature, pH and dissolved oxygen. The major factor for the expression of lipase activity has always been reported as the carbon source, since lipases are inducible enzymes. The ability of lipases to perform very specific biochemical transformations has made them increasingly popular in food, detergent, cosmetic, organic synthesis and pharmaceutical industries **(Hasan *et al.*, 2006)**.

The use of agro-industrial residues as substrates for lipase production undoubtedly favors the reduction of production costs and pollution associated with these substrates. Considering the above facts, production of lipase from whey is a pertinent area of research and this study encompasses optimization of the production process using response surface methodology. It enables effective medium optimization using a limited number of trials and has been widely employed for the optimization of a number of production processes. It has been successfully applied for lipase production as well **(Gupta *et al.*, 2007; Saxena and Saxena, 2004; Vohra and Satyanarayana, 2002)**.

The objective of our study was to isolate bacterial strains from soil sample and screen them for lipase activity and lactose utilization. The best isolate was subjected to optimization of lipase production using One-Factor-at-a-Time (OFAT) and Response Surface Methodologies (RSM).

MATERIAL AND METHODS

Chemicals

Bovine serum albumin (BSA) was purchased from SIGMA-ALDRICH, USA. Microbiological media components and all other chemicals were of reagent grade and procured from HI-MEDIA, India.

Isolation of bacterial strains

Bacterial strains were isolated from soil sample exposed to the effluent discharged from Aavin Dairy, located in Coimbatore District of India. They were serially diluted and spread plates from the dilutions were made with nutrient agar.

Screening for lipase activity

Primary screening

Nutrient agar medium was supplemented with 0.01% (w/v) $CaCl_2.H_2O$. Tween 80 autoclaved at 120°C for 20 min was added to the molten agar medium at 45°C to give a final concentration of 1.0%. The medium was shaken well until the Tween 80 had dissolved completely, poured in Petri dishes and pure cultures of the bacterial isolates were streaked on it. An opaque halo around the colonies indicates production of lipase or esterase.

Secondary screening

Culture medium containing 0.8% nutrient broth, 0.4% NaCl and 1.75% agar-agar was adjusted to pH 7.0 and autoclaved. Then, 1ml of filter sterilized Rhodamine B solution (1mg/ml in distilled water) was added per 100ml of medium, followed by the addition of sterilized olive oil, to make a final concentration of 2.5%. The medium was poured in Petri dishes, circular wells of 3.0mm diameter were punched in the agar and 1.0ml of the culture supernatant was dispensed in each well. Lipase activity was identified on the plate after 24h of incubation at 37°C, as an orange fluorescent halo surrounding the colonies under UV illumination at 350 nm **(Kouker & Jaeger, 1987)**.

Lactose utilization by the isolates

Phenol red lactose broth was prepared by dissolving 1g of lactose monohydrate in nutrient broth followed by the addition of phenol red indicator (pH 7.6), making a final concentration of 0.002%. The lipase producing isolates were then inoculated in the broth and incubated at 37°C. The results were recorded at the 24^{th} and 48^{th} hours of incubation. A colour change to yellow indicates positive result for lactose utilization, while colour change to magenta or hot pink indicates negative result for the test.

Lipase production and quantification of activity

The isolates showing positive results for lipase activity and lactose utilization were subjected to submerged fermentation in a production medium which contained 2% (v/v) olive oil as an inducer. After 24h incubation, the cells were harvested by centrifuging at 10000 g for 10 min and the supernatant was used as the crude enzyme extract and its lipase activity was determined using titrimetric assay based on the procedure of **Jensen (1983)**.
In this assay, activity was determined by titration with 0.05 N NaOH, which measures the concentration of free fatty acids released. This method uses an emulsion composed of 25% olive oil and 75% arabic gum (7% p/v) as the substrate. The reaction was conducted in 125-ml Erlenmeyer flasks with 5ml of emulsion, 2ml of 0.1 M phosphate buffer pH 7 and 1ml of crude lipase. The Erlenmeyer flasks were incubated for 30 min at 45°C in a shaker incubator (ORBITEK, India). The reaction was quenched with 10ml solution of acetone and ethanol (1:1). The fatty acids released during the reaction were titrated in the presence of phenolphthalein as an indicator. Lipase activity was expressed in enzyme units (U/ml), which correspond to 1µmol of fatty acids released per minute under the specified assay conditions.

Identification and characterization of the lipase producers

Cultural characteristics of the isolates were observed in nutrient agar plates and cellular characteristics were noted by suitable staining techniques. Important biochemical characteristics were also studied **(Cappuccino & Sherman, 2013)**. The best lipase producer was further identified by MALDI-TOF Mass Spectroscopy.

Collection and characterisation of whey

Whey was collected in 1L containers from 'Organic Cheese-Making Farm Stay', a cottage industry in Coonoor (Nilgiris District, India). It was stored under refrigerated conditions in the lab until further processing. The carbohydrate (lactose) content in the whey was measured by estimating the amount of reducing sugar using the Dinitrosalicylic Acid (DNS) method **(Miller, 1959)**. This method was employed since lactose is the only sugar that is present in whey. Protein estimation was carried out using Bovine Serum Albumin (BSA) as the standard **(Lowry *et al.*, 1951)**.

Medium optimization for lipase production

One-Factor-at-a-Time

OFAT method was employed to study the effects of different inducers, nitrogen sources, mineral salts and whey concentrations on production of lipase by the best isolate. In this study, various concentrations of whey (25%, 50%, 75% and 100%) were tried in the basal medium for lipase production. Subsequently, this medium was supplemented with different oil sources (coconut, olive, castor, sunflower, groundnut, gingelly and palm oils). Various organic nitrogen sources (peptone, yeast extract, tryptone, peptone and beef extract) were then added individually to the basal medium. Several mineral salts (NaCl, $CaCl_2$, $MgSO_4$, $MnSO_4$, $NH_4H_2PO_4$, K_2HPO_4, KH_2PO_4, $CuSO_4$, $ZnSO_4$ and Na_2MoO_4) were also screened. These experiments were done in 250ml Erlenmeyer flasks containing 100ml culture medium of pH 7.0, incubated at 37ºC in an orbital shaker for 3 days.

Response Surface Methodology

RSM was adopted for statistical optimization of the culture medium for lipase production. Its main advantage is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results. In this study, Box-Behnken design with four independent variables was employed to find out the optimum medium composition for lipase production. It is an independent, rotatable quadratic design with no embedded factorial or fractional factorial points. In this *design*, the treatment *combinations* are at the midpoints of edges of the process *space* and at the *centre* **(Box & Behnken, 1960)**. This method involves the application of statistical experimental design at two different levels and a centre point. It is useful for building second-order surface models in bioprocess development and optimization, resulting in maximum yield.
Based on the results of OFAT, the independent variables and their ranges were chosen. Their coded and actual levels are given in Table 1.

Table 1 Independent variables and their levels in Box-Behnken design

Variables	Symbols	Coded & actual levels		
		-1	**0**	**+1**
Whey % (v/v)	A	50	75	100
Sunflower oil % (v/v)	B	2	4	6
Beef extract % (w/v)	C	0.25	0.625	1.0
Calcium chloride % (w/v)	D	0.1	0.2	0.3

The coding of the variables could be expressed by the following equation:

$$x_i = (X_i - X_z) / \Delta X_i$$

where x_i is the dimensionless value of an independent variable; X_i is the real value of an independent variable; X_z is the real value of an independent variable at the centre point; and ΔX_i is the step change in the real value of the variable i corresponding to a variation of a unit for the dimensionless value of the variable. Design and data analyses were carried out using DESIGN EXPERT statistical software (version 9.0.3.1, Stat-Ease, Inc., Minneapolis, USA). Analysis of variance (ANOVA) was done to validate the optimization process. The optimal medium composition was estimated through regression analysis and three-dimensional response surface plots of the independent variables.

RESULTS AND DISCUSSION

Bacterial isolation

Soil sample collected from the dairy industry premises was serially diluted and the higher dilutions (10^{-4} - 10^{-8}) were spread on nutrient agar and incubated for 24 h. From these plates, 43 bacterial isolates were identified based on visible differences in colony morphology. They were maintained as pure cultures in agar slants and stored at -4°C for further studies.

Screening for lipase activity

The isolates were initially screened for their ability to produce lipase or esterase by Tween 80 agar plate assay. Among the 43 isolates, 28 showed significant zone of lipid hydrolysis after 24h of incubation. These isolates from the primary screening were tested for true lipase activity in Rhodamine olive oil agar secondary screening. The formation of orange fluorescent halo under UV light due to the reaction of hydrolysed substrate with Rhodamine B confirms the production of lipase. In this, 26 isolates displayed true lipase activity.

Lactose utilization by the isolates

The lipase producing isolates were tested for lactose utilization in order to ascertain their ability to grow in whey based media. In the phenol red lactose broth test used for this purpose, two of the isolates (strains B3 and B10) were

found to be positive. Phenol red is an indicator, which below pH 6.8 turns to yellow and above 8.4 turns to hot pink or magenta. The isolates which have the ability to metabolise lactose reduce the pH of the medium and the indicator changes the color of the broth to yellow.

Lipase production and quantification of activity

The two isolates B3 and B10 capable of lactose utilization in the phenol red lactose broth test were cultivated by submerged fermentation in a production medium supplemented with 2% olive oil for selection of the best lipase producer. The strain B10 produced 0.79U/ml of lipase after the incubation period of 48h, while B3 was capable of producing only 0.67U/ml (Figure 1). Hence, B10 was chosen for further studies on the optimization of enzyme production.

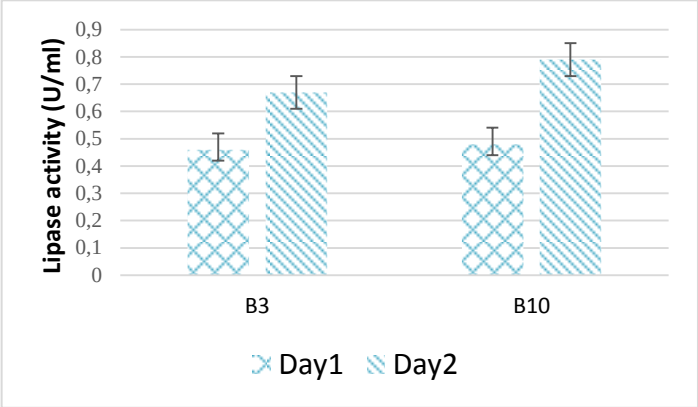

Figure 1 Lipase activity of the bacterial isolates B3 and B10

Identification and characterization of the lipase producers

The cellular, cultural and biochemical characteristics of the lipase producers were studied and the results for strain B10 are furnished in Table 2, based on which it was inferred as belonging to *Bacillus* sp. The organism was a Gram positive, motile and endospore forming rod. On nutrient agar, it produced large, cream-colored, irregular and flat colonies with undulate margins. It was able to ferment the sugars glucose, sucrose, mannitol and lactose (ONPG test). It was capable of citrate utilization and catalase production. However, it showed variable results for malonate utilization and Voges Proskauer tests. Its identity was confirmed by MALDI-TOF Mass Spectroscopy (Table 3). In this analysis, the match pattern of the sample was studied using the Bruker taxonomy tree **(Aitken, 2005)**. Ten closest matches were considered and the culture was identified as *Bacillus subtilis.*

Table 2 Cellular, cultural and biochemical characteristics of strain B10

Cellular characteristics		**Biochemical characteristics**	
Cell morphology	Rod	Glucose fermentation	+
Gram reaction	+	Sucrose fermentation	+
Endospore	+	Arabinose fermentation	-
Motility	+	Mannitol fermentation	+
Cultural characteristics		Trehalose fermentation	-
Size	Large	Malonate fermentation	+/-
Color	Creamy	Voges Proskauer test	+/-
Form	Irregular	Citrate utilization	+
Margin	Undulate	Arginine utilization	-
		ONPG test	+
Elevation	Flat	Nitrate reduction	-
		Catalase production	+

+ Positive result; - Negative result; +/- Variable result.

Biochemical characterisation of whey

Whey contains lactose, a reducing sugar, as the only carbohydrate and it was estimated by the DNS method. The lactose content of whey was recorded as 39.5±0.3 g/L. The total protein content was assayed by Lowry's method and found to be to 5.4±0.5 g/L. These values seem to be agreeable with the results reported by **Mawson (1994)**. However, the composition of whey tends to change with the characteristics of the milk sample used.

Medium optimization for lipase production

OFAT method

OFAT method was used in the initial part of the optimization study to understand the impacts of inducers (oil source), nitrogen sources, mineral salts and whey concentrations on lipase production. Various concentrations of whey were taken in the basal medium for producing lipase. Organic nitrogen sources (5.0g/L) were added individually to this basal medium. Different inducing oils (2% v/v) and mineral salts (0.1% v/v) were also screened, the results of which have been discussed in this section.

Table 3 MALDI-TOF MS identification of strain B10

Rank (Quality)	**Matched Pattern**	**Score value**	**NCBI identifier**
1 (-)	*Bacillus subtilis* ssp. *subtilis* DSM 10T DSM	2.116	135461
2 (-)	*Bacillus subtilis* ssp. *subtilis* DSM 5660 DSM	2.041	135461
3 (-)	*Bacillus subtilis* DSM 5611 DSM	2.001	1423
4 (-)	*Bacillus subtilis* DSM 5552 DSM	1.978	1423
5 (-)	*Bacillus mojavensis* DSM 9205T DSM	1.833	72360
6 (-)	*Bacillus vallismortis* DSM 11031T DSM	1.687	72361
7 (-)	*Bacillus subtilis* ssp. *spizizenii* DSM 15029T DSM	1.677	96241
8 (-)	*Bacillus atrophaeus* DSM 675 DSM	1.523	1452
9 (-)	*Bacillus atrophaeus* DSM 2277 DSM	1.398	1452
10 (-)	*Bacillus subtilis* 107_W_7_QSA IBS	1.376	1423

Effect of whey concentration

The isolate B10 was inoculated in 250ml Erlenmeyer flasks containing 100ml whey of different concentrations (25, 50, 75 and 100%) and supplemented with 2% olive oil added as an inducer. Lipase assay was carried out with the cell-free supernatant after 3 days of incubation, from which 75% whey was found to be optimum for the production of lipase by the organism (Figure 2). In medium containing 100% whey, increased biomass production was seen, but the enzyme activity was lower. Many organisms are not capable of utilizing the lactose present in whey and in their study **Palanisamy *et al.* (2016)** have reported lactose to be the least favored carbon source for lipase production by *Bacillus amyloliquefaciens* PS35.

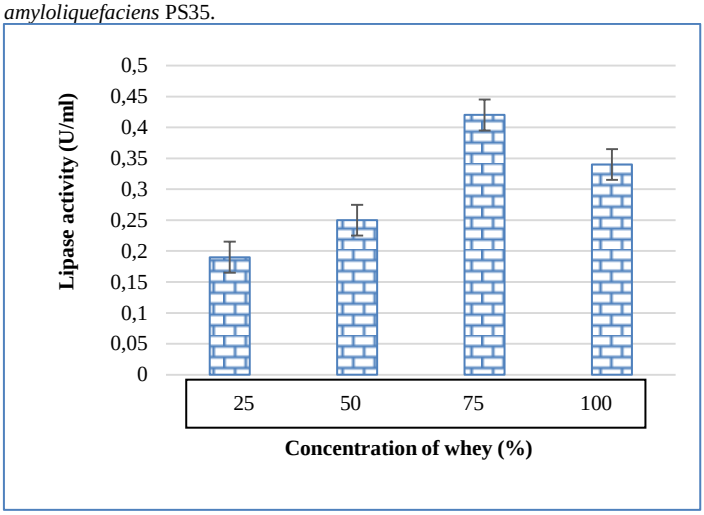

Figure 2 Effect of whey concentration on lipase production

Effect of oil sources

The effects of different oil sources in inducing lipase production was deciphered by culturing the isolate B10 in media containing 75% whey and supplemented with 2% of oil source (coconut, olive, castor, sunflower, groundnut, gingelly or palm oil) added as inducer. Appreciable lipase production was achieved in the presence of all the inducers tested. Maximum activity of 0.54U/ml was seen in the medium supplemented with sunflower oil (Figure 3). This explains that the sunflower oil acts as a good inducer in stimulating the production of lipase. In other studies too, sunflower oil has been shown to have the optimum inducing effect on lipase production by *Bacillus* sp. **(Palanisamy *et al.*, 2016)**. Triacylglycerols, fatty acids and hydrolysable esters also induce lipase production by *Bacillus* sp. **(Gupta N *et al.*, 2004)**.

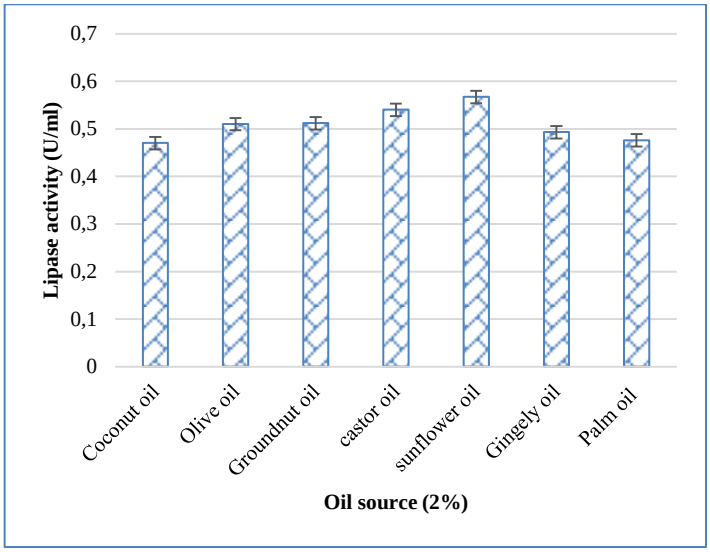

Figure 3 Effect of different oil sources in inducing lipase production

Effect of nitrogen sources

The lipolytic isolate was then cultivated in Erlenmeyer flasks containing 75% whey supplemented with 2% of sunflower oil and 5g/L of nitrogen source (yeast extract, beef extract, malt extract, peptone or tryptone). Maximum lipase activity of 0.69U/ml was witnessed in the medium having beef extract as the nitrogen source (Figure 4). In another such study, lipolytic titers in the culture medium of *Bacillus* sp. have been found to increase in the presence of NH_4Cl and casein **(Gupta R *et al.*, 2004)**.

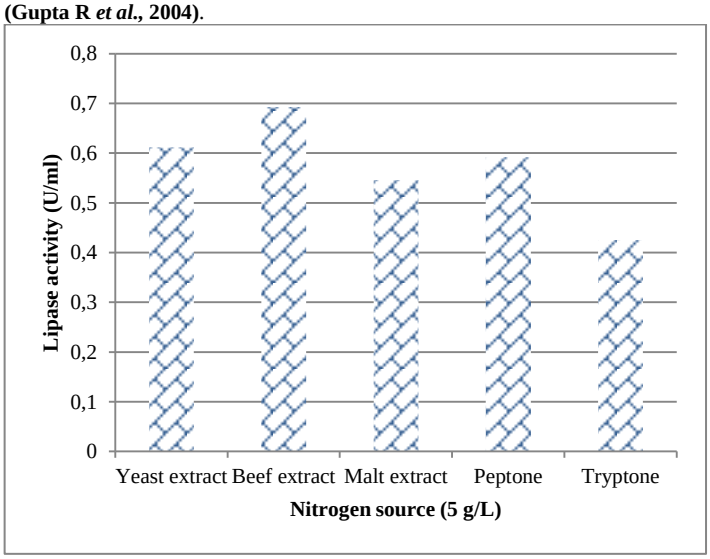

Figure 4 Effect of different nitrogen sources on lipase production

Effect of mineral salts

In order to judge the effects of different mineral salts on lipase production by the isolate, it was inoculated in media containing 75% whey, 2% sunflower oil and 5g/L beef extract, to which different mineral salts (0.1%) were individually added. When lipase assay was carried out at the end of the 3-day incubation period, highest activity of 0.67U/ml was recorded in the culture medium containing $CaCl_2$ (Figure 5). Lipase production has been widely reported to be induced by the presence of $CaCl_2$. For instance, lipase activity in *Burkholderia multivorans* has been positively influenced by the presence of Ca^{2+} ions in the production medium **(Gupta *et al.*, 2007)**.

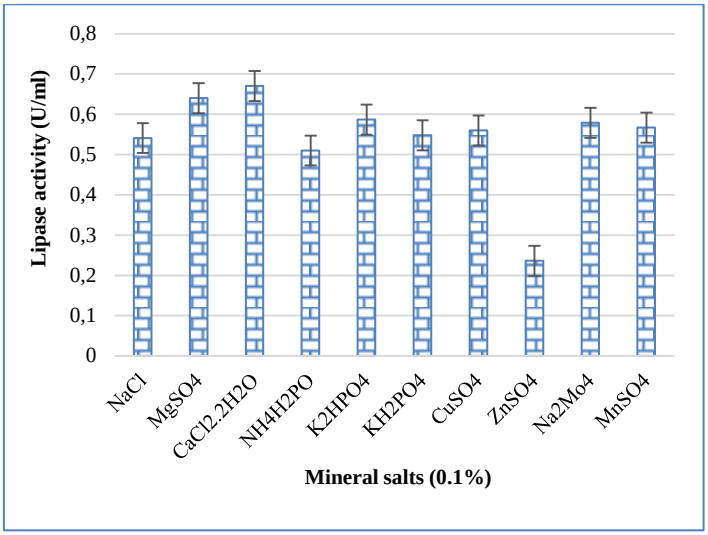

Figure 5 Effect of mineral salts on lipase production

Response surface methodology

Based on results from the preceding OFAT studies, the medium for statistical optimization consisted of whey, beef extract, sunflower oil and $CaCl_2$. Their concentrations were optimized and interaction effects studied through the Box-Behnken Design of RSM, for maximizing the lipase production by *Bacillus subtilis* B10. It involved 29 experimental runs including 5 centre points and the experiments were carried out in triplicates. After the incubation period, lipase assay was performed using the cell-free supernatant and enzyme activity was recorded as the response. The experimental design and results from the investigation are listed in Table 4, showing the mean observed responses (R) for the model.

Table 4 RSM experimental design and responses obtained

Trial	Factor A (Whey) (% v/v)	Factor B (Sunflower oil) (% v/v)	Factor C (Beef extract) (% w/v)	Factor D (Mineral salts) (% w/v)	Response Lipase activity (U/ml)
1	100	2	0.625	0.2	0.79
2	75	4	0.625	0.2	0.934
3	50	4	1	0.2	0.75
4	100	4	0.625	0.1	0.817
5	50	4	0.625	0.3	0.756
6	75	2	0.625	0.1	0.772
7	75	4	0.625	0.2	0.954
8	75	4	0.625	0.2	0.943
9	75	6	0.25	0.2	0.824
10	100	4	0.625	0.3	0.856
11	50	2	0.625	0.2	0.64
12	75	4	0.25	0.1	0.847
13	75	4	0.25	0.3	0.894
14	100	6	0.625	0.2	0.749
15	75	6	0.625	0.3	0.856
16	75	2	0.625	0.3	0.816
17	50	6	0.625	0.2	0.68
18	50	4	0.25	0.2	0.742
19	50	4	0.625	0.1	0.692
20	75	4	0.625	0.2	0.945
21	100	4	0.25	0.2	0.838
22	75	4	0.625	0.2	0.947
23	75	4	1	0.3	0.93
24	75	2	0.25	0.2	0.798
25	75	4	1	0.1	0.862
26	75	6	1	0.2	0.85
27	75	2	1	0.2	0.789
28	100	4	1	0.2	0.85
29	75	6	0.625	0.1	0.833

ANOVA for the response surface quadratic model is provided in Table 5.

Table 5 ANOVA for response surface quadratic model

Source	Sum of squares	Degrees of freedom	Mean square	F value	p-value Prob>F
Model	0.19	14	0.014	69.66	< 0.0001
A	0.034	1	0.034	173.22	< 0.0001
B	2.914E-003	1	2.914E-003	14.79	0.0018
C	6.453E-004	1	6.453E-004	3.27	0.0919
D	6.769E-003	1	6.769E-003	34.35	< 0.0001
AB	1.640E-003	1	1.640E-003	8.32	0.0120
AC	4.000E-006	1	4.000E-006	0.020	0.8887
AD	1.562E-004	1	1.562E-004	0.79	0.3883
BC	3.062E-004	1	3.062E-004	1.55	0.2330
BD	1.102E-004	1	1.102E-004	0.56	0.4668
CD	1.102E-004	1	1.102E-004	0.56	0.4668
A^2	0.11	1	0.11	544.31	< 0.0001
B^2	0.064	1	0.064	322.40	< 0.001
C^2	4.673E-003	1	4.673E-003	23.72	0.0002
D^2	6.732E-003	1	6.732E-003	34.16	< 0.0001
Residual	2.759E-003	14	1.971E-004		
Lack of Fit	2.550E-003	10	2.550E-004	4.87	0.0702
Pure Error	2.092E-004	4	5.230E-005		
Cor Total	0.19	28			

ANOVA showed F-value of 69.66, which implies that the model is highly significant. There is only a 0.01% chance that F-value this high could occur due to noise. p-values less than 0.0500 indicate that the corresponding model terms are significant. In this case, A, B, D, AB, A^2, B^2, C^2 and D^2 are significant model terms. Values greater than 0.1000 specify that the model terms are insignificant. In general, larger magnitudes of t, F and smaller p-values indicate that the corresponding coefficient terms are significant. The R^2 value gives a measure of how much variability in the observed response could be explained by the experimental parameters and their interactions. The closer the value of R^2 is to 1, the better the correlation between the experimental and predicted values **(Reddy *et al.*, 2007)**. R^2 value of 0.9745 for the quadratic model implies that the model predicts the reponse well.

The following second order polynomial equation shows the empirical relationship between the independent variables and the response:

$U = +0.94 + 0.053 \times A + 0.016 \times B + 7.333E\text{-}003 \times C + 0.024 \times D - 0.020 \times AB + (1.000E\text{-}003 \times AC) - (6.250E\text{-}003 \times AD) + (8.750E\text{-}003 \times BC) - (5.250E\text{-}003 \times BD) + (5.250E\text{-}003 \times CD) - 0.13 \times A^2 - 0.099 \times B^2 - 0.027 \times C^2 - 0.032 \times D^2$

Where, U = Lipase activity (U/ml), A, B, C and D denote Whey, Sunflower oil, Beef extract and Calcium chloride, respectively. This equation could be used to make predictions about the response for given levels of each of the factors. They are also useful for identifying the relative impacts of the factors by comparing the factor coefficients.

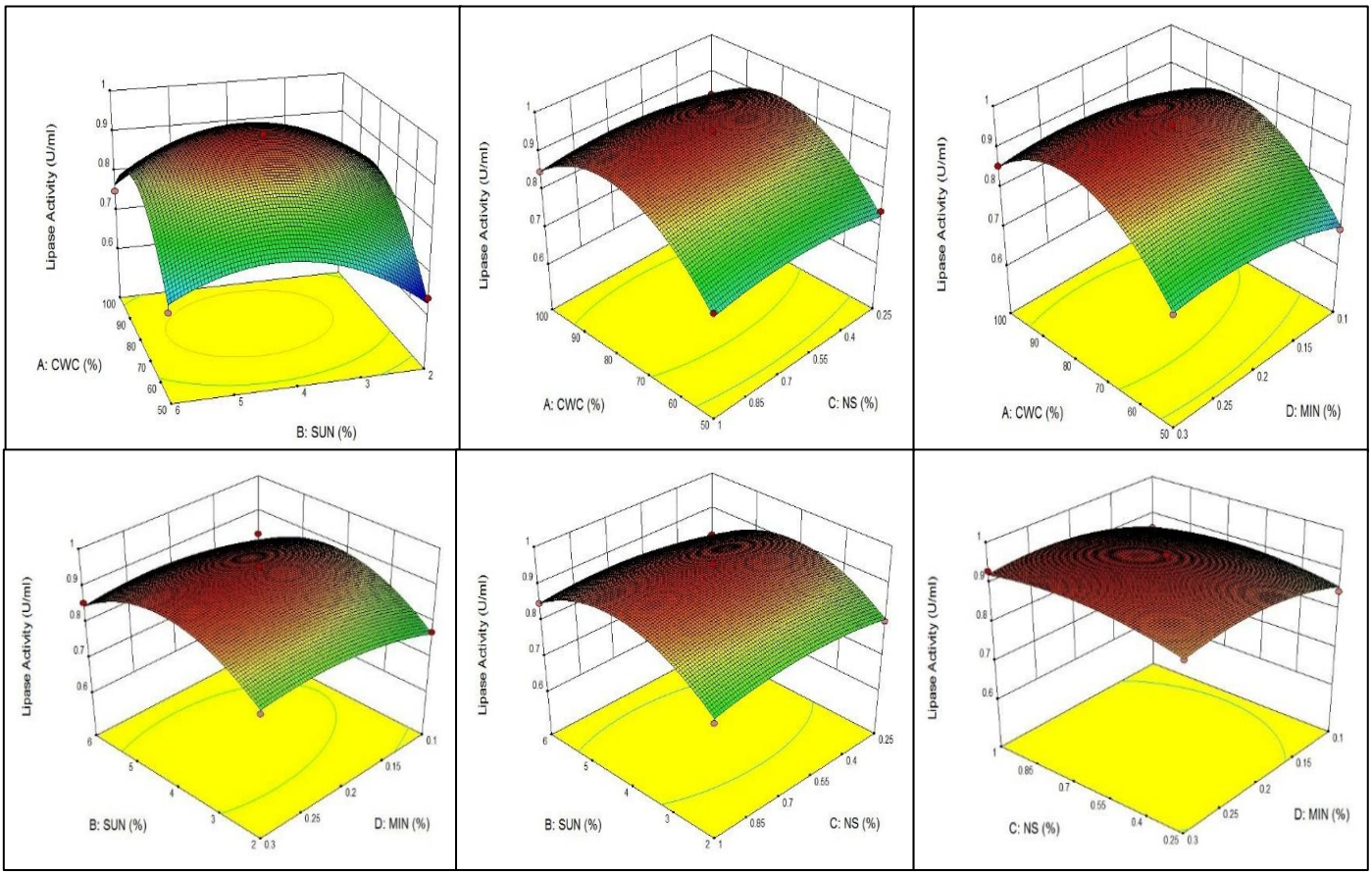

Figure 6 Response surface plots showing the interactions between independent variables and their effects on lipase activity

The contour and 3D plots are ways of expressing the regression equation graphically. They depict the interactions among the variables and are used to determine the optimum concentration of each factor for good response. Response surface plots representing the effects of process variables and their interactions on lipase activity (response) were obtained from the software, as shown in Figure 6.

According to the Box–Behnken design, the optimum conditions for achieving maximal lipase production using *B. subtilis* B10 are as follows: Whey - 75% (v/v); sunflower oil - 4% (v/v), beef extract - 0.625% (w/v); and $CaCl_2$ - 0.2% (w/v). Based on the optimum values, a desirability ramp was developed using numerical optimization techniques. The experiments were performed in triplicate and the results were compared with predicted values from the model equation (Table 6). The maximum lipase activity obtained under the optimum conditions was observed to be 0.917U/ml. The mean of the experimentally obtained lipase activity was compared with the predicted one specified by the model. Good correlation between the two values indicates that the Box–Behnken design could be effectively applied to optimize the process for lipase production from *B. subtilis* B10 using whey as substrate.

Table 6 Predicted and experimental values of lipase activity under the optimum conditions

Optimum conditions				Lipase activity (U/ml)	
Whey (% v/v)	Sunflower oil (% v/v)	Beef extract % (w/v)	$CaCl_2$ % (w/v)	Predicted value	Experimental value
75	4	0.625	0.2	0.945	0.917±0.024

In literature, lipase production from *Geobacillus thermoleovorans* has been optimized by applying D-optimal design **(Sanchez-Otero *et al.*, 2011)**. Statistical experimental design has also been used to optimize the production of extracellular lipase from *Stenotrophomonas maltophilia* CCR11 **(Hasan-Beikdashti *et al.*, 2012)**. Response surface methodology has facilitated the improvement of lipase production from *Burkholderia sp.* C20 **(Liu *et al.*, 2006)**.

CONCLUSION

This work has led to the isolation of promising bacterial strains from soil samples exposed to dairy industrial waste discharge. Screening for lipase activity has established the lipase producing potentials of 26 isolates, 2 of which were capable of growth in culture medium containing lactose as the sole carbon source. Experimentation of whey-based media for optimization of lipase production has identified beef extract, sunflower oil and $CaCl_2$ as ideal constituents for supplementing the culture medium. Statistical optimization has shed light on the optimal concentrations and interaction effects of key variables that are conducive for enhanced lipase production. Applying the Box-Behnken design of RSM has resulted in a maximum lipase activity of 0.954U/ml in a culture medium containing 75% (v/v) whey, 4% (v/v) sunflower oil, 0.625% (w/v) beef extract and 0.2% (w/v) $CaCl_2$. Whey being an industrial waste stream whose disposal poses significant environmental threat, its utilization as a substrate in the production of an industrially valuable enzyme could be viewed as offering the benefit of offsetting environmental pollution. Since it is not a valuable resource but a waste product, its use also serves as a practically advantageous cost-cutting measure in the production process.

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

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