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Enhancing Extracellular Lipolytic Enzyme Production In An Arctic Bacterium, *Psychrobacter* sp. ArcL13, By Using Statistical Optimization And Fed-Batch Fermentation

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A strain isolated from seawater samples in the Chuckchi Sea and exhibiting extracellular lipolytic activity was identified using 16S rRNA gene sequence analysis as *Psychrobacter sp*. ArcL13. The lipolytic enzyme exhibited cold-active properties and high hydrolytic activity toward *p*-nitrophenyl caprylate (C_8), *p*-nitrophenyl decanoate (C_{10}), and sunflower oil. Statistical optimization of the medium components was performed to enhance the production of cold-active extracellular lipolytic activity. Glucose, yeast extract (YE), and NaCl were selected as the main efficient nutrient sources. Fed-batch fermentation using optimized medium with concentrated YE as the main feeding material showed a maximum lipolytic activity of 10.7 U/mL, which was a 21-fold increase in production over unoptimized flask culture conditions. The information obtained in the present study could prove applicable to the production of cold-active lipase on a large scale.

Keywords *arctic bacterium, fed-batch fermentation, lipolytic activity, psychrophile, statistical optimization*

INTRODUCTION

Bacteria, yeasts, algae, and fungi in cold environments have developed various adaptations that enable them to compensate for the adverse efforts of low temperature.[1] Cold-adapted microorganisms are the source of cold-active enzymes that might be used for biotechnological exploitation because of their high catalytic activity at low temperatures and their low thermostability.[1,2] Furthermore, cold-active enzymes are expected to be useful in different fields.[1,3,4]

Lipolytic enzymes, including lipases (triacylglycerol hydrolases, EC 3.1.1.3), catalyze the hydrolysis and synthesis of triglycerides, other fatty acid esters, short acids, and fairly complex acids. These enzymes are produced by animals, plants, fungi, and microorganisms.[5–7] Microbial lipases have the potential for commercial applications due to their stability, broad

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substrate specificity, position specificity, and chiral selectivity.[7–10] Some lipases show high organic solvent tolerance and stability, and their activity relies on the presence of an oil–water interface.[11] Lipases are very useful enzymes because they have industrial applications in the food industry, chemical compound synthesis, detergent industry, biopolymers and biodiesel synthesis, medical and pharmaceutical applications, and the cosmetic industry.[12–14] Several cold-adapted, lipase-producing, psychrophilic bacteria have been isolated, and lipolytic enzymes isolated from psychrophilic bacteria have been studied.[12]

Recently, some bacterial strains producing extracellular lipolytic enzymes were isolated from the Chuckchi Sea of the Arctic Ocean. Among them, *Psychrobacter* sp. showed the highest extracellular lipolytic activity. The current study is focused on investigating the effects of medium composition on production of lipolytic activity, and on finding efficient production conditions using this microorganism. Classical medium optimization by changing one independent variable at a time and keeping other factors constant is time-consuming and has the limitation of ignoring interactions among the factors. However, statistical experimental design, including two-factorial design and response surface methodology, has proven effective for the optimization of culture medium.[15–19] Therefore, statistical experimental design was carried out to optimize the medium components. This study describes a statistical approach and fed-batch fermentation to optimize and enhance the production of lipolytic activity in bacterial strains isolated from the Arctic Sea. The properties of the lipolytic activity were also analyzed.

MATERIALS AND METHODS

Screening and Selection of Lipase-Producing Bacteria

Bacterial strains were isolated from seawater samples collected in the Chuckchi Sea of the Arctic. Each sample was spread on Marine Broth plates containing 1% glyceryl tributyrate (Sigma, St. Louis, MO). Plates were incubated at 15°C for 5 days, and bacteria having extracellular lipolytic activity were selected by measuring the clear zone surrounding the colony. In total, 13 bacterial strains were selected and tested for lipolytic activity using liquid cultures (Table 1). Thirteen strains isolated from the Arctic sea were characterized using 16S rRNA sequencing. The sequences were aligned using the EzTaxon program (www.eztaxon.org)[20] and the phylogenetic tree was constructed using Phydit version 3.2.[21] Supernatants from 13 cultures were separated by centrifugation and their activities were determined by assaying their *p*-nitrophenyl caprylate (pNPC) hydrolysis rates.

Strain and Media

Psychrobacter sp. ArcL13 (KCTC 12498BP) was selected as the lipase-producing bacterium after comparing the extracellular lipolytic activities of 13 bacterial strains. The seed culture was prepared by inoculating a flask containing Marine Broth (MB) medium from a glycerol stock and incubating for 24 hr at 15°C. The seed culture (10%) was transferred to the main culture medium for cell growth and lipolytic enzyme production. Unoptimized Marine Broth (MB) medium contained the following (g/L): peptone, 5; yeast extract, 1; $Fe(C_6H_5O_7)$, 0.1; NaCl, 19.45; $MgCl_2$, 5.9; Na_2SO_4, 3.24; $CaCl_2$, 1.8; KCl, 0.55; $NaHCO_3$, 0.16; KBr, 0.08; along with

TABLE 1
Sampling Sites of the 13 Bacteria From Chuckchi Sea

Sample code	GPS	Source
ArcL1	N 75.31.24 W 178.47.04	Multi core
ArcL2	N 74.59.99 W 175.49.98	Bongo net
ArcL4	N 75.20.69 W 173.45.97	Box core
ArcL5	N 75.31.25 W 178.47.04	Box core
ArcL6	N 75.31.25 W 178.47.04	Box core
ArcL7	N 77.04.53 W 172.19.61	Multi core
ArcL8	N 74.59.99 W 175.49.98	Bongo net
ArcL9	N 75.22.27 W 177.17.44	Bongo net
ArcL10	N 75.22.27 W 177.17.44	Bongo net
ArcL11	N 74.37.05 W 166.23.77	CTD membrane
ArcL12	N 74.37.05 W 166.23.77	CTD membrane
ArcL13	N 74.18.00 W 162.29.99	CTD membrane
ArcL14	N 75.20.69 W 173.45.97	CTD membrane

Note. Samples were collected during the 2012 KOPRI-led Arctic Araon scientific cruise (August 1 to September 10, 2012).

1 mL/L trace metal solution, which is composed of $FePO_4$, 10.0 g/L; $SrCl_2$, 34.0 g/L; H_3BO_3, 22.0 g/L; Na_2SiO_3, 4.0 g/L; NaF, 2.4 g/L; NH_4NO_3, 1.6 g/L; and Na_2HPO_4, 8.0 g/L.

Characterization of Lipolytic Enzyme From *Psychrobacter* sp. ArcL13

Hydrolysis rates toward various synthetic substrates (pNP-butyrate, pNP-caprylate, pNP-decanoate, and pNP-dodecanoate) were measured via the typical spectrophotometric method at 25°C. A different assay method was used for pNP-dodecanoate and pNP-palmitate as follows. Ten microliters of enzyme solution was added to 890 μL of reaction buffer containing 50 m*M* of Tris-HCl (pH 8.0), 0.1% gum arabic, and 0.2% deoxycholate. The reaction was initiated by adding 100 μL of 8 m*M* substrate in isopropanol, and stopped by the addition of 0.5 mL of 3 *M* HCl. After centrifugation, 333 μL of supernatant was mixed with 1 mL of 2 *M* NaOH, and the absorbance at 405 nm was measured.[9] Hydrolysis rates of lipolytic enzyme toward vegetable oils, including olive oil and soybean oil, were measured using the pH-stat method.[22]

The apparent optimum temperature for lipolytic activity was determined in the standard activity assay at various temperatures ranging from 0°C to 60°C using *p*-nitrophenyl caprylate as the substrate. The thermal stability of the enzyme was measured by incubating the enzyme in 50 mM Tris-HCl buffer (pH 8.0) at various temperatures ranging from 0°C to 70°C for 0.5 hr. After 0.5 hr, each sample was assayed for lipolytic activity using *p*-nitrophenyl caprylate as the substrate at 25°C.

The optimal pH for lipolytic activity was determined in the standard activity assay at variable pH ranging from 6 to 10 by using *p*-nitrophenyl caprylate as the substrate. The pH stability of the enzyme was determined by measuring residual lipolytic activity after incubating the enzyme at 4°C for 24 hr in the following buffers: phosphate-buffered saline (PBS; pH 6, 6.5, 7, 7.45); 50 m*M* Tris-HCl (pH 7.4, 8, 8.5, 9); and 50 m*M* glycine-NaOH (pH 9, 9.5, 10).

Selection of Optimal Carbon and Nitrogen Sources

The effect of the carbon source on liquid cultures of *Psychrobacter* sp. ArcL13 was studied using 10 g/L of each of the following carbon sources: glucose, galactose, fructose, lactose, sucrose, maltose, and glycerol. In experiments to determine the best complex nitrogen source, 6 g/L of peptone, yeast extract, tryptone, soy peptone, or tryptic soy broth was added to the ZoBell medium instead of 5 g/L of yeast extract and 1 g/L of peptone.

Statistical Optimization

The Plackett–Burman design[23] was used to identify the essential components significantly influencing the production of lipolytic activity from *Psychrobacter* sp. ArcL13. Based on the design, 11 components of the medium were examined at two levels, low (−) and high (+), resulting in a first-order model, $\mathbf{Y} = \beta_0 + \Sigma\beta_i\mathbf{X}_i$, where **Y** is the predicted response (lipolytic activity), β_0 is the model intercept, β_i is the linear coefficient, and $\mathbf{X}_i$ is the level of the independent variable.

To optimize the concentration of each component selected using the Plackett–Burman design, a surface response method called the Box–Behnken design[24] was applied. The amount of each component was coded into three levels: (−), (0), and (+) for low, intermediate, and high concentrations, respectively. For the prediction of the optimal concentrations, a second-order polynomial model was designed to describe the relationship between the independent variables (components) and the response: $\mathbf{Y} = \beta_0 + \Sigma\beta_i\mathbf{X_i} + \Sigma\beta_{ij}\mathbf{X_iX_j} + \Sigma\beta_{ii}\mathbf{X_i^2}$, where **Y** is the predicted response (lipolytic activity), and β_0, β_i, β_{ij}, and β_{ii} are the constant and regression coefficients of the model, with $\mathbf{X}_i$ and $\mathbf{X}_j$ representing the independent nutrient.

Fed-Batch Fermentations

Fed-batch cultures were carried out in a 5-L jar fermenter (Minifors, Infors HT, Switzerland) at 15°C using optimized medium. The cells were allowed to grow for 40 hr in batch culture and then a DO (dissolved oxygen)-stat fed-batch culture was started using 120 g/L of YE, 20 g/L of glucose, and 35 g/L of NaCl as the feeding solution. The DO set-point for the initiation of the DO-stat fed-batch culture was 30% air saturation under atmospheric pressure. The feed rate was increased automatically in an exponential fashion.

Cell Growth Monitoring and Assay of Lipolytic Activity

The growth of *Psychrobacter* sp. ArcL13 was monitored by measuring the optical density of the culture at 600 nm using a spectrophotometer (S-3100, Scinco, Korea). The lipolytic activity in the broth was measured using pNPC as the substrate.[22] The reaction mixture consisted of 10 μL of 10 mM substrate in acetonitrile, 40 μL of ethanol, and 10–200 μL of enzyme solution, made up with 50 m*M* Tris-HCl buffer (pH 8.0) to 1 mL. The amount of *p*-nitrophenoxide formed by the enzyme was determined by measuring the absorbance at 405 nm and using

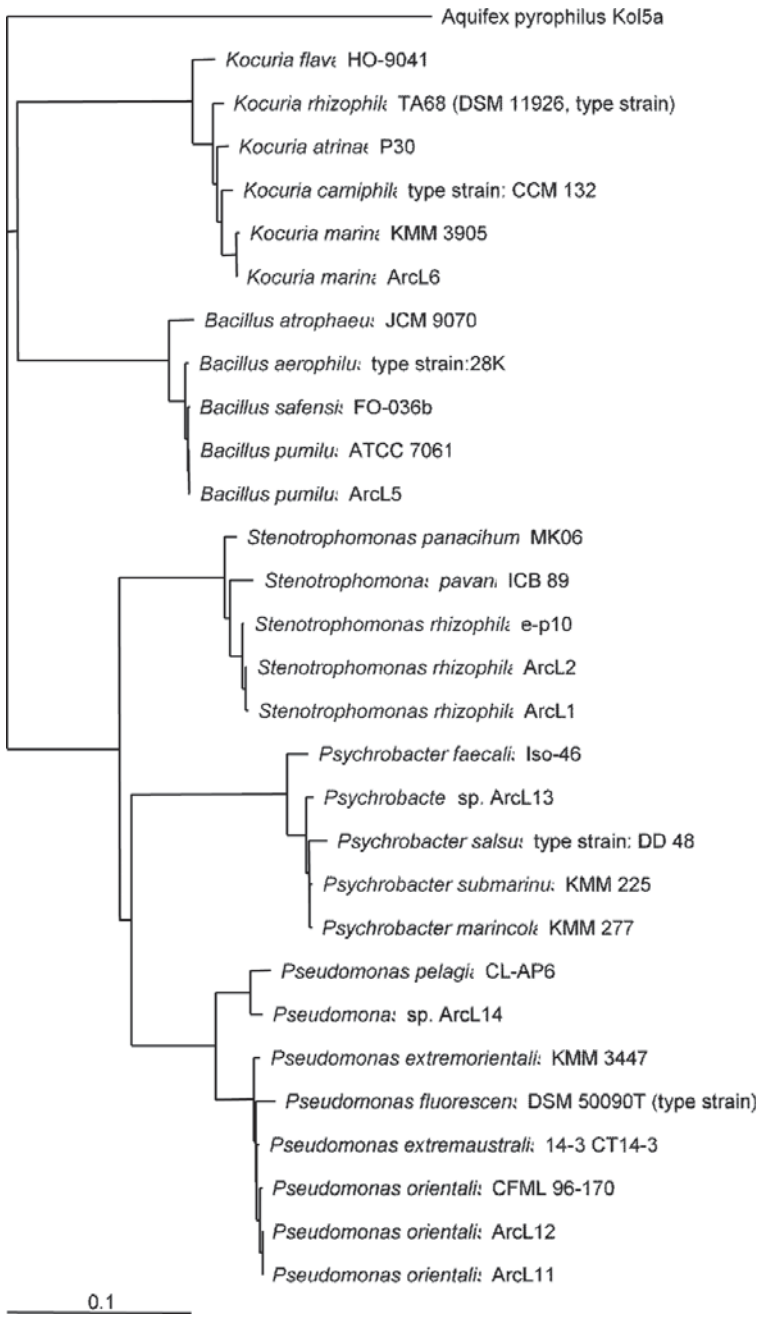

FIGURE 1 Phylogenetic relationship based on 16S rRNA sequences of isolates. 16S rRNA gene sequence analysis showed that strains 1 and 2 (=4, 7, 8, 9, and 10) were *Stenotrophomonas rhizophila*, 5 was *Bacillus pumilus*, 6 was *Kocuria marina*, 11 and 12 were *Pseudomonas orientalis*, 13 was *Psychrobacter* sp., and 14 was *Pseudomonas* sp. The distance scale means the number of differences between sequences.

the extinction coefficient $17{,}700\,M^{-1}\,\mathrm{cm}^{-1}$. The unit of activity per milliliter ($1\,\mathrm{U/mL}$) was defined as the amount needed to hydrolyze $1\,\mu\mathrm{mol}$ substrate to *p*-nitrophenoxide per minute per culture volume.

RESULTS

Phylogenetic Relationship and Lipolytic Activities of Lipase-Producing Bacteria

Thirteen strains isolated from the Arctic sea were characterized using 16S rRNA sequencing. The sequences were aligned using the EzTaxon program (www.eztaxon.org)[20] and the phylogenetic

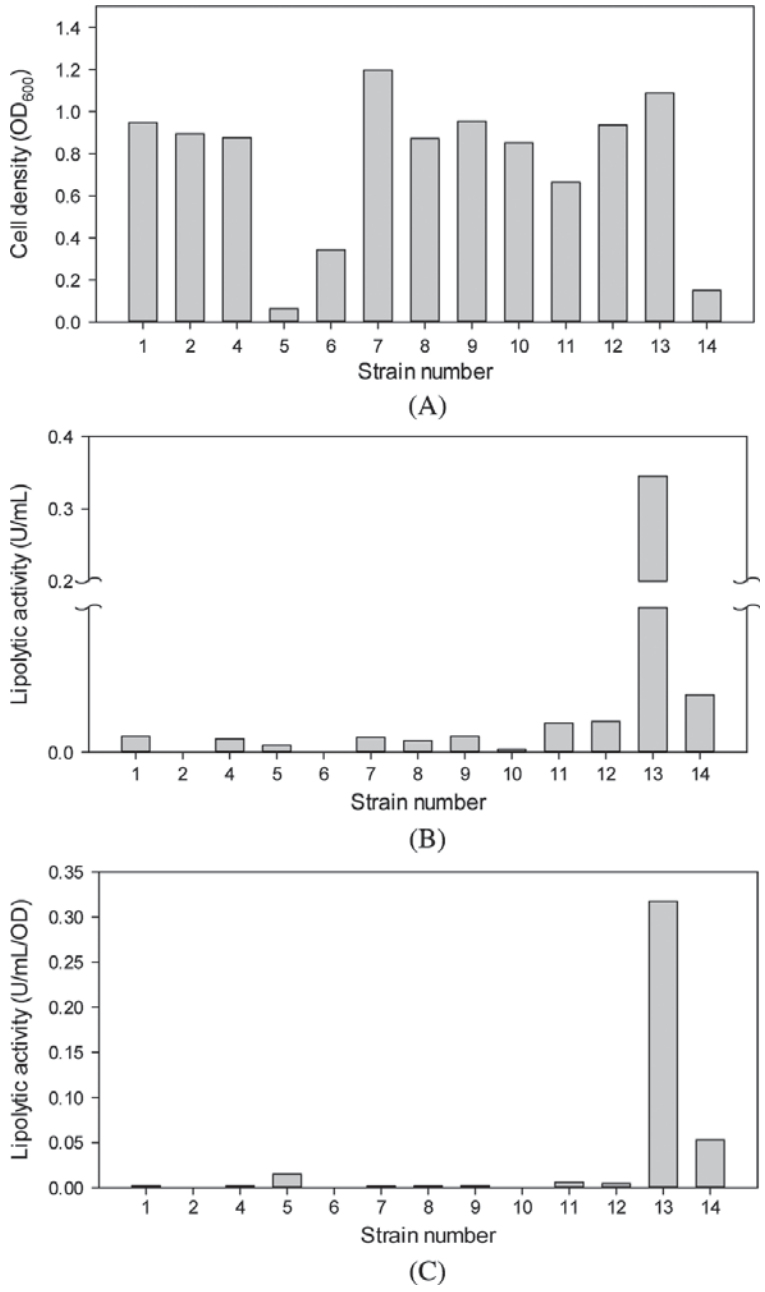

FIGURE 2 Comparison of lipolytic enzyme production from the 13 bacterial strains. Cell densities (A) and lipolytic activities (B) were measured using 24-hr cultures in MB medium at 15°C. The normalized lipolytic activity (C) also calculated.

tree was constructed using Phydit version 3.2.[21] Figure 1 shows that isolates ArcL1 and 2 were *Stenotrophomonas rhizophila*, ArcL5 was *Bacillus pumilus*, ArcL6 was *Kocuria marina*, ArcL11 and 12 were *Pseudomonas orientalis*, ArcL13 was *Psychrobacter* sp., and ArcL14 was *Pseudomonas* sp. These strains were cultured in MB medium and their supernatants were used as the lipolytic enzyme sources. Extracellular lipolytic activities were measured using the pNPC assay

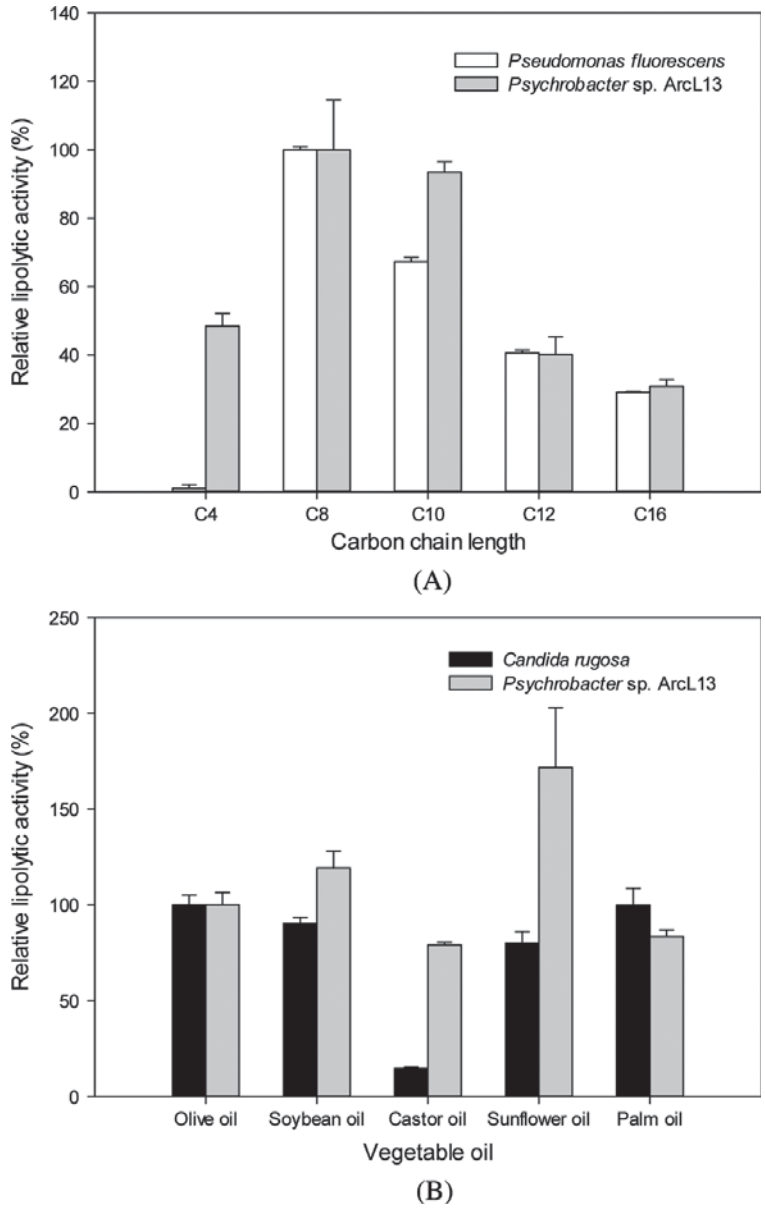

FIGURE 3 Substrate specificities of lipolytic enzymes. Hydrolytic activities of lipases (*Pseudomonas fluorescens*, white bar; *Psychlobacter* sp. ArcL13, gray bar) toward various *p*-nitrophenyl esters (C_4, C_8 [100%], C_{10}, C_{12}, and C_{16}) (A), and hydrolytic activities of lipases (*Candida rugosa*, black bar; *Psychlobacter* sp. ArcL13, gray bar) toward vegetable oils (olive oil [100%], soybean oil, castor oil, sunflower oil, palm oil) (B) were measured. Error bars show the standard deviation derived from three independent experiments.

method, and the *Psychrobacter* sp. ArcL13 strain showed the highest activity (0.35 U/mL) among them (Figure 2).

Characterization of Lipolytic Activity From *Psychrobacter* sp. Arcl13

Substrate specificity was measured spectrophotometerically using various lengths of *p*-nitrophenyl esters as substrates at pH 8.0 and 25°C, such as *p*-nitrophenyl butyrate (pNPB; C_4), *p*-nitrophenyl caprylate (pNPC; C_8), *p*-nitrophenyl decanoate (pNPD; C_{10}), *p*-nitrophenyl dodecanoate (pNPD; C_{12}), and *p*-nitrophenyl palmitate (pNPP; C_{16}). As shown in Figure 3A, lipases had the highest hydrolytic activity toward *p*-nitrophenyl caprylate (C_8). Lipolytic enzyme from *Psychrobacter* sp. ArcL13 had higher activity toward a short-chain substrate (C_4) in comparison with lipase from *Pseudomonas fluorescens* (Sigma). The lipolytic enzyme from *Psychrobacter* sp. ArcL13 hydrolyzed vegetable oils, and showed higher activity toward sunflower oil and soybean oil than toward olive oil (Figure 3B).

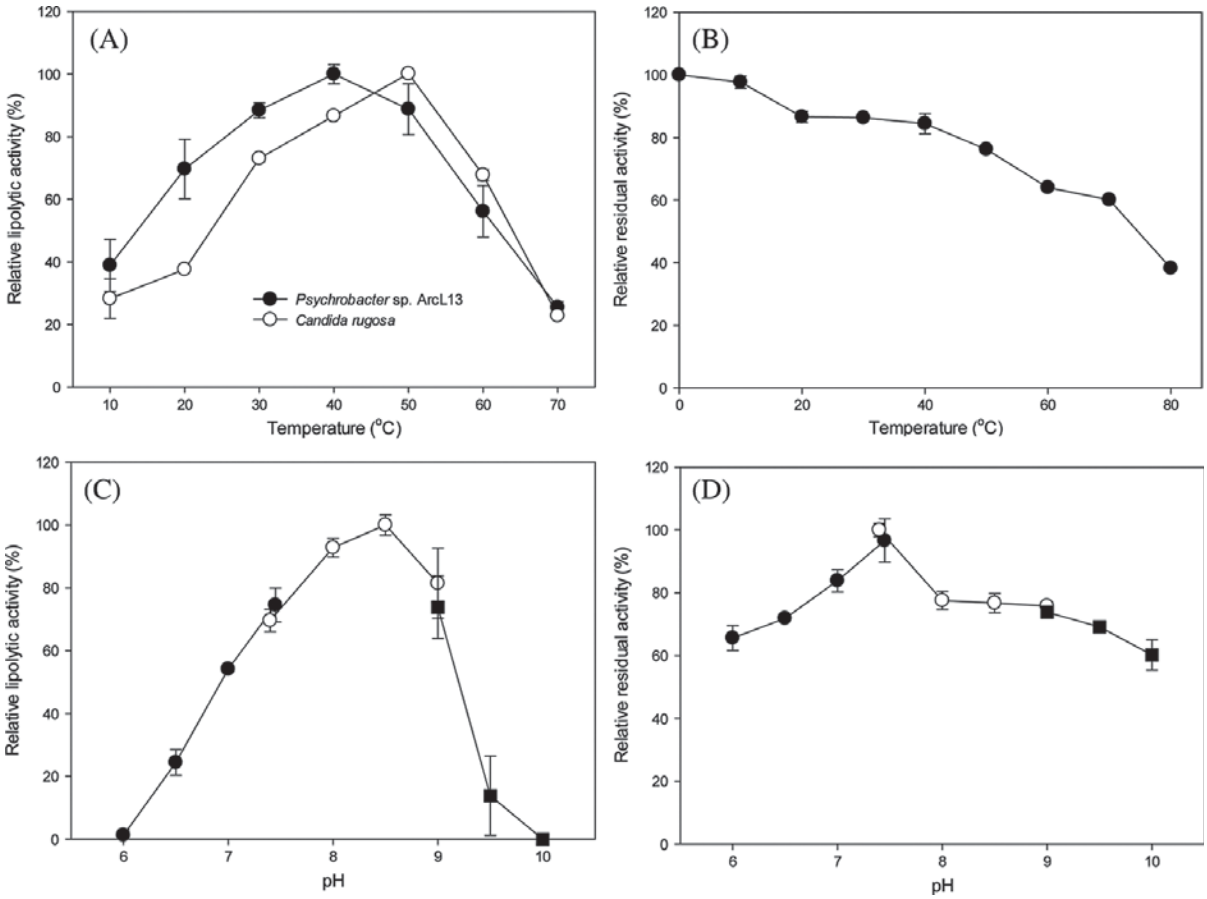

FIGURE 4 Effects of temperature and pH on lipolytic activity. Lipolytic activities were assayed at various temperatures (A) (●, semipurified enzyme; ○, *Candida rugosa*) and pH buffers (C) (●, PBS; ○, 50 m*M* Tris-HCl; ■, 50 m*M* glycine-NaOH). Lipolytic enzyme was incubated at different temperatures (B) and pH buffers (D) (●, PBS; ○, 50 m*M* Tris-HCl; ■, 50 m*M* glycine-NaOH). Error bars show the standard deviation derived from three independent experiments.

Lipolytic activity was analyzed at various temperatures (0–80°C). The lipolytic enzyme from ArcL13 showed maximum activity at 40°C toward *p*-nitrophenyl caprylate, when assayed at pH 8.0, and exhibited 39% and 70% of its highest activity at 10°C and 20°C, respectively (Figure 4A). Other cold-adapted lipases show high activity at 20–40°C[3]; thus, the lipolytic enzyme from *Psychrobacter* sp. ArcL13 was expected to be a typical cold-adapted enzyme. The thermostability of the culture media was determined by preincubating the enzyme at various temperatures (0–80°C) for 0.5 hr, and then the remaining activity was determined at 25°C. When the lipolytic enzyme was incubated at 40°C, the residual activity maintained 84% of maximal activity. When the lipolytic enzyme was incubated at 50°C for 0.5 hr, the activity slowly decreased (Figure 4B).

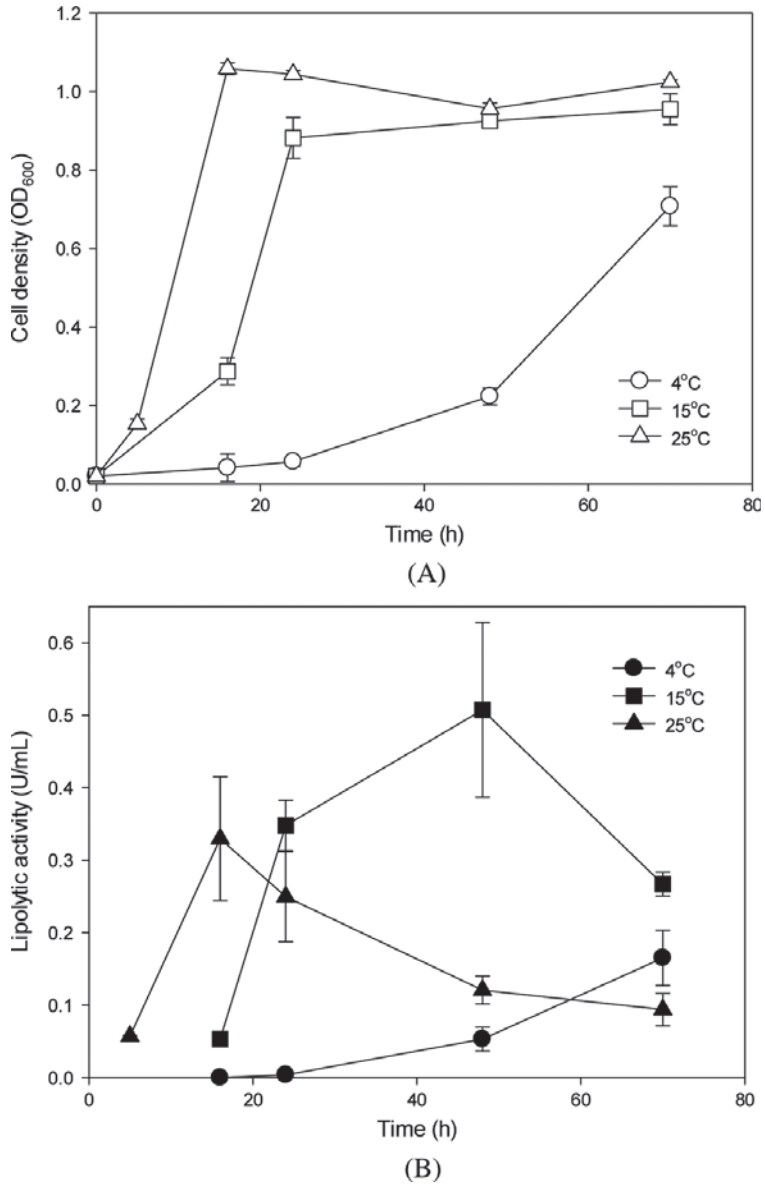

FIGURE 5 Effect of culture temperature on (A) cell growth and (B) lipolytic activity. Cells were grown at 4°C (circles), 15°C (squares), and 25°C (triangles) in MB medium.

The effect of pH on activity was examined in the pH range 6.0 to 10.0 using *p*-nitrophenyl caprylate as the substrate. The lipolytic enzyme from *Psychrobacter* sp. ArcL13 showed maximum activity at pH 8.5 (Figure 4C). The effect of pH on stability was determined by pre-incubating the enzyme in the pH range 6.0 to 10.0 at 4°C for 1 hr, and then the remaining activity was determined at pH 8.0 using *p*-nitrophenyl caprylate as the substrate. The culture medium was stable at the pH range 7.0 to 7.5, retaining more than 80% residual activity (Figure 4D).

Effect of Culture Temperature

As shown in Figure 5, cell growth depends on the culture temperature, and the maximal specific growth rates were 0.06, 0.17, and 0.41 hr^{-1} at 4, 15, and 25°C, respectively. However, the

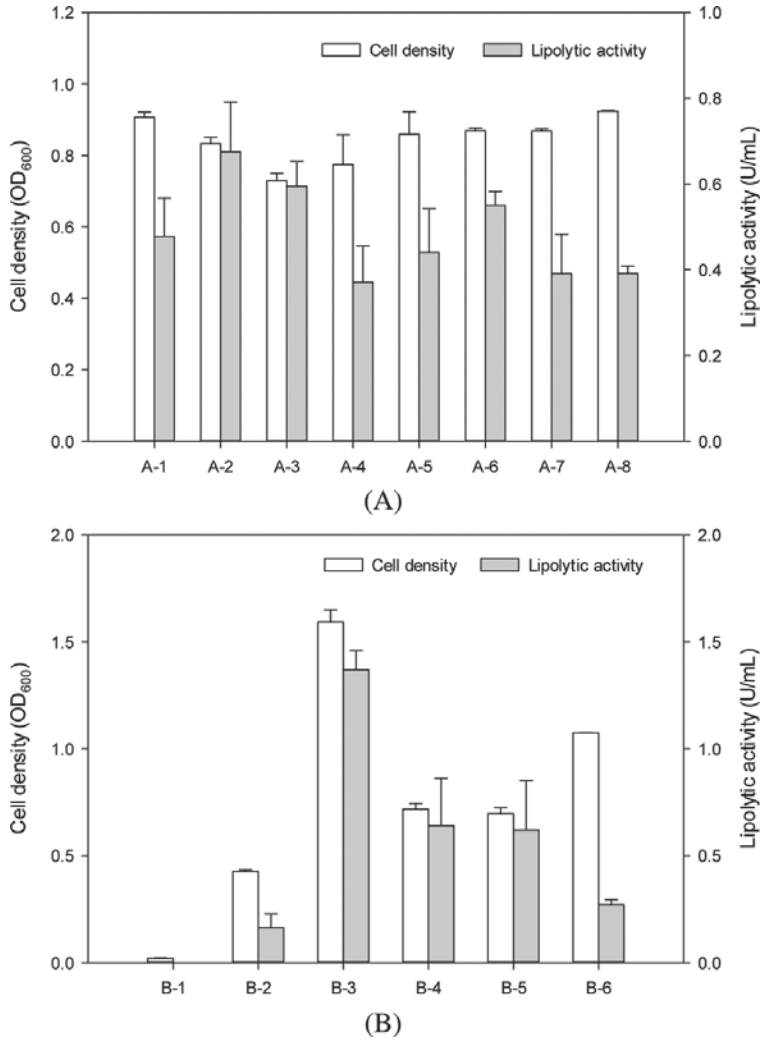

FIGURE 6 Effect of (A) carbon sources and (B) nitrogen sources on cell growth and lipolytic enzyme production. (A) Cells were grown in MB medium containing 10 g/L of different carbon sources: A-1, control (not added); A-2, glucose; A-3, galactose; A-4, fructose; A-5, sucrose; A-6, lactose; A-7, maltose; A-8, glycerol. (B): Cells were grown in MB medium containing glucose (10 g/L) and 6 g/L of different nitrogen sources: B-1, control (not added); B-2, peptone; B-3, yeast extract; B-4, soytone; B-5, tryptone; B-6, casamino acid. After 24 hr of cultivation at 15°C, cell density (white bar) and lipolytic activity (gray bar) were measured.

lipolytic activity reached a maximum level of 0.50 U/mL when cells were grown at 15°C for 48 hr. These results indicate that the optimal growth temperature for lipolytic activity production was 15°C.

Determination of Best Carbon and Nitrogen Sources

To identify a suitable carbon source for lipolytic enzyme production by flask cultivation of *Psychrobacter* sp. ArcL13, 10 g/L of different carbon sources were tested. Most carbon sources had a negative effect on final cell density, while glycerol had a slightly positive effect (Figure 6A). Glucose, galactose, and lactose showed a positive effect, whereas the other carbon sources had inhibitory effects on lipolytic enzyme production. When glucose was used as the carbon source, lipolytic activity was 0.67 U/mL, representing about 1.9-fold higher enzyme activity than that seen in MB medium.

The production of lipolytic enzyme was conducted in the presence of different complex nitrogen sources (6 g/L), including peptone, yeast extract, soytone, tryptone, and casamino acid, in MB medium containing glucose (10 g/L) instead of peptone and YE. The lipolytic activity reached a maximum value of 1.36 U/mL with 6 g YE per liter (Figure 6B). The effect of YE on lipolytic activity was sufficient for it to be chosen as a complex nitrogen source.

Selection of Significant Components in Medium

To eliminate unnecessary components for lipolytic activity, each of the 11 different elements in the medium was tested. Table 2 shows the test levels, effect, *t*-statistics, and *p*-value for each medium component. Medium components showing a *p*-value of < 0.05 were accepted as significant factors affecting the production of lipolytic enzyme. It was found that the *p*-values of $Fe(C_6H_5O_7)$, Na_2SO_4, $CaCl_2$, $NaHCO_3$, and trace elements were >0.05, indicating that these five components were not significant factors. Six components, namely, NaCl, $MgCl_2$, KCl, KBr,

TABLE 2
Statistical Analysis of Medium Components Using the Initial Plackett–Burman Experiment

Variable	Medium component	High (+) value (g/L)	Low (−) value (g/L)	Effect	*t*-Statistic	*P*-Value
X_1	$Fe(C_6H_5O_7)$	0.1	0	0.0585	0.59	0.567
X_2	NaCl	19.45	0	0.2554	2.57	0.024
X_3	$MgCl_2$	8.8	0	0.2847	2.87	0.014
X_4	Na_2SO_4	3.24	0	0.089	0.90	0.388
X_5	$CaCl_2$	1.8	0	0.2056	2.07	0.061
X_6	KCl	0.55	0	0.4364	4.39	0.001
X_7	$NaHCO_3$	0.16	0	0.0325	0.33	0.749
X_8	KBr	0.08	0	0.3554	3.58	0.004
X_9	Trace	0.082	0	0.1121	1.13	0.281
X_{10}	Glucose	10	0	0.2655	2.67	0.020
X_{11}	Yeast extract	6	0	1.4407	14.51	0.000

TABLE 3
Statistical Analysis of Selected Medium Components Using the Secondary Plackett–Burman Design

Variable	Medium component	High (+) value (g/L)	Low (−) value (g/L)	Effect	*t*-Statistic	*P*-Value
X_1	NaCl	38.9	3.89	−0.3843	−2.49	0.030
X_2	$MgCl_2$	17.6	1.76	−0.2714	−1.75	0.107
X_3	KCl	1.1	0.11	−0.0266	−0.17	0.866
X_4	KBr	0.16	0.016	0.0336	0.22	0.832
X_5	Glucose	20	2.0	0.3538	2.29	0.043
X_6	Yeast extract	12	1.2	1.0304	6.66	0.000

glucose, and yeast extract, showed positive effects on lipolytic enzyme production, with *p*-values of <0.05, and these components were retested using a secondary Plackett–Burman design. Among them, NaCl, glucose, and yeast extract were selected as the most effective components for the production of lipolytic enzyme (Table 3).

Optimization of Medium Components for Lipolytic Activity

The significant components for lipolytic enzyme production were studied further using a Box–Behnken design. Table 4 shows the medium components tested for Box–Behnken optimization and their values, which were calculated by linear multiple regression using Minitab software. As a result, the following equation was obtained: $Y = 4.873 - 0.209X_1 - 0.232X_2 + 1.099X_3 - 0.331X_1^2 - 0.018X_2^2 - 3.030X_3^2 - 0.140X_1X_2 - 0.072X_1X_3 + 0.158X_2X_3$, where Y is the predicted response (lipolytic activity), and X_1, X_2, and X_3 are the concentrations of NaCl, glucose, and yeast extract, respectively. The main effect and interaction effects of the three factors, NaCl, glucose, and yeast extract, at different concentrations are shown in Figure 7. The expected lipolytic activity was determined to be 5.1 U/mL at the optimal NaCl, glucose, and yeast extract concentrations of 7.6, 4.0, and 23.1 g/L, respectively, representing about 10-fold higher enzyme activity due to optimization.

Fed-Batch Fermentation Using Optimized Medium

To confirm our predictions and optimized components, lipase activity from *Psychrobacter* sp. ArcL13 was measured in different media: MB medium, carbon- and nitrogen-source-optimized

TABLE 4
Box–Behnken Optimization of Selected Significant Medium Components

Variable	Medium component	High (+) value (g/L)	Center (0) value (g/L)	Low (−) value (g/L)
X_1	NaCl	20	11	2
X_2	Glucose	40	22	4
X_3	Yeast extract	40	22	4

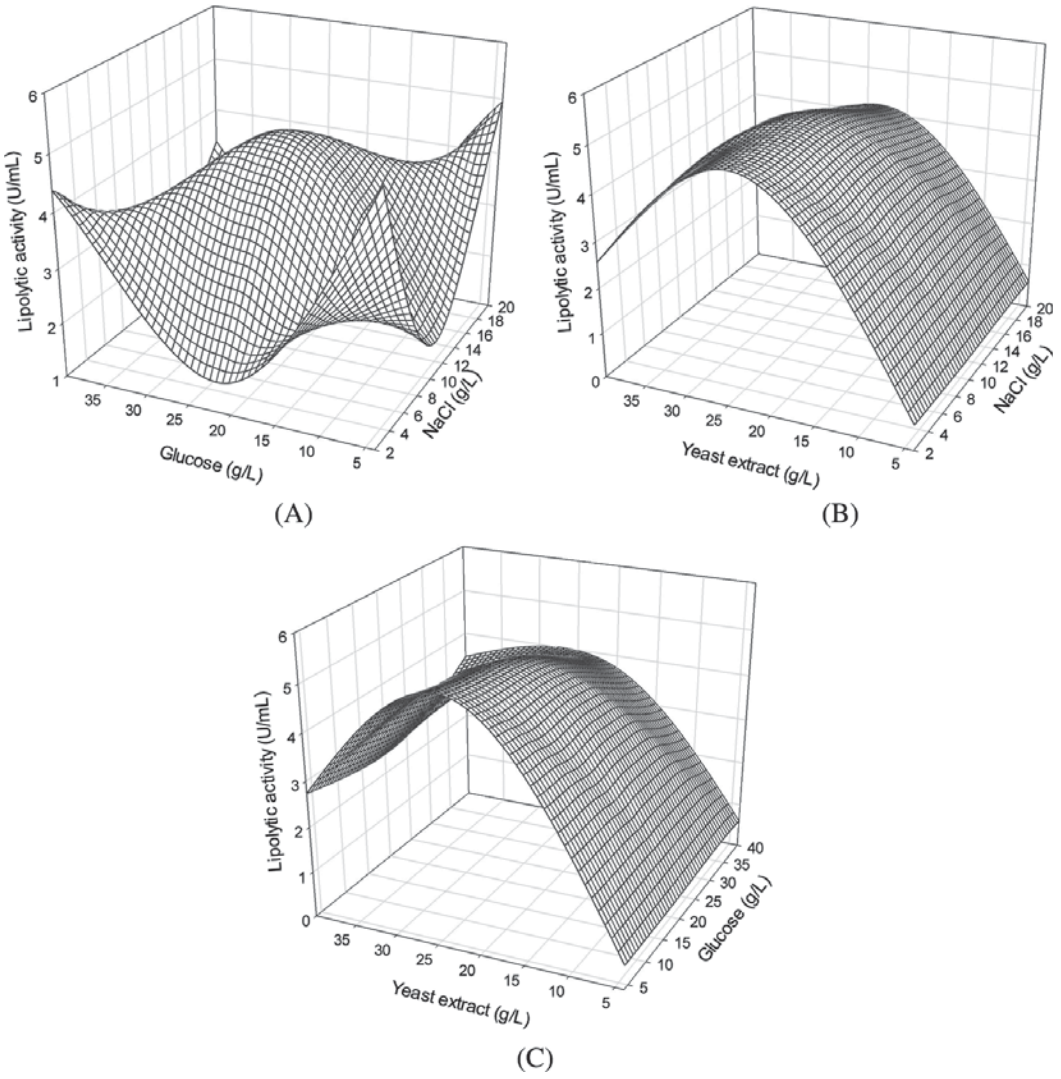

FIGURE 7 Three-dimensional response plot showing the effect of (A) glucose and NaCl; (B) yeast extract and NaCl; and (C) yeast extract and glucose on lipolytic enzyme production.

medium, and optimized medium following the Box–Behnken design. Figure 8A shows the time profiles of lipase activity using different media and the same operation conditions. Maximal lipase production values of 0.49, 1.34, and 5.18 U/mL were attained in MB medium, carbon- and nitrogen-source-optimized medium, and optimized medium following the Box–Behnken design, respectively. These results indicate that optimization enhanced lipase activity and the model adequately predicted lipase activity. To elevate the production of lipolytic enzyme, fed-batch fermentation was carried out in a 5-L jar fermenter using optimized medium. Upon feeding NaCl, glucose, and yeast extract, maximal lipolytic activity increased to 10.7 U/mL, which was two times higher than that from batch culture (Figure 8B). Lipase activity was associated with cell growth, and the cell density reached a maximal OD_{600} value of 13.5 after 96 hr of incubation.

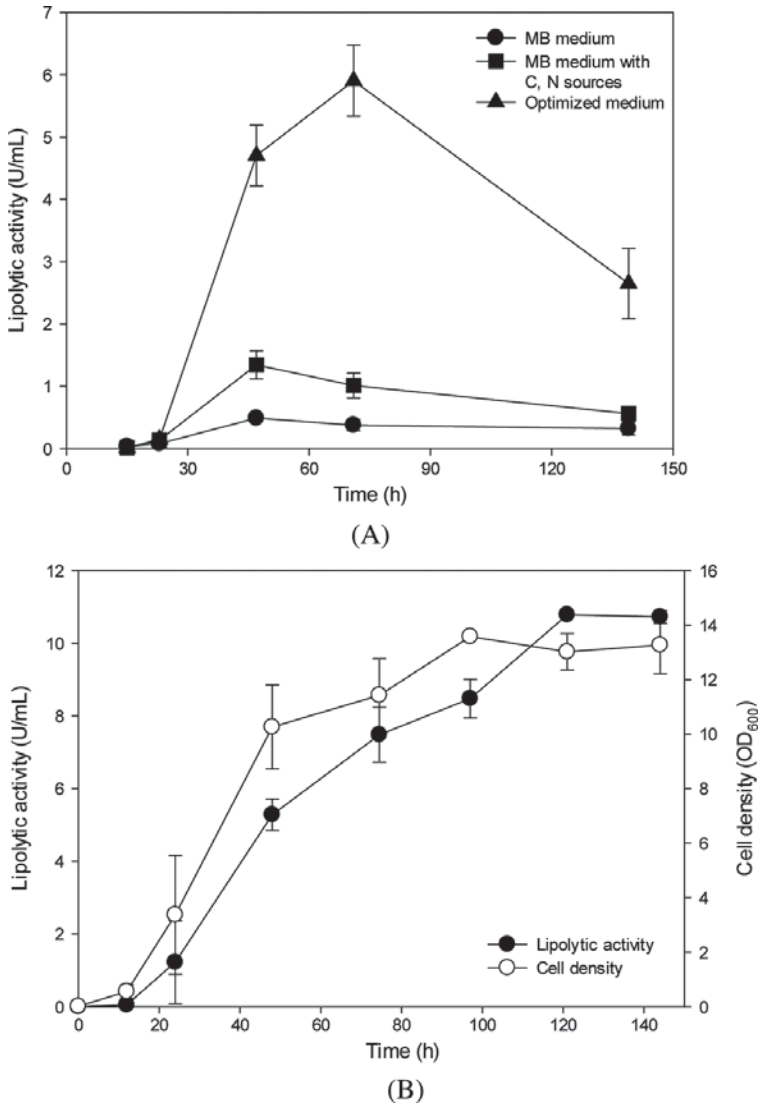

FIGURE 8 Time profile of lipolytic enzyme production in (A) batch fermentation (●, MB medium; ■, MB medium with carbon and nitrogen sources; ▲, optimized medium) and (B) fed-batch fermentation (○, cell density; ●, lipolytic activity).

DISCUSSION

To enhance the productivity of the lipolytic enzyme originally found in the Arctic microorganism *Psychrobacter* sp. ArcL13, optimized conditions were investigated. The lipolytic enzyme from *Psychrobacter* sp. ArcL13 showed high activity toward short- and medium-chain fatty acids (C_8–C_{10}), similar to that derived from Antarctic *Bacillus*.[22] Because the strain was derived from the Chuckchi Sea of the Arctic and the optimum temperature for the production of lipolytic enzyme was 15°C, the optimal temperature for lipolytic activity was almost the same as that seen for other psychrophilic lipases from *Bacillus pumilus*,[22] *Photobacterium lipolyticum* sp.,[9] and *Psychrobacter* sp.[2] These results confirm that the lipolytic enzyme from *Psychrobacter* sp.

ArcL13 had cold-active properties. The lipolytic enzyme showed high activity at weakly alkaline conditions and the activity rapidly decreased at acidic conditions. However, the enzyme was very stable at alkaline conditions.

In general, each lipase-producing organism has its own requirement with respect to special conditions for maximal lipase production.[25–28] The medium components affecting the production of lipolytic enzyme were optimized using statistical design. There have been several reports that some complex nitrogen sources stimulate lipase production: soybean meal,[17] tryptone,[16] yeast extract,[25] and peptone.[27] In our case, yeast extract showed the most positive effect on lipolytic enzyme production. The lipolytic activity when yeast extract was included in the medium reached 1.36 U/mL, which was 10-fold higher than when peptone was included instead of yeast extract (Figure 6B). A statistical approach was found to be very effective for optimizing medium components in a manageable number of experimental runs. The final optimized medium composition was as follows (g/L): glucose, 4.0; yeast extract, 23.1; $Fe(C_6H_5O_7)$, 0.1; NaCl, 7.6; $MgCl_2$, 8.8; Na_2SO_4, 3.24; $CaCl_2$, 1.8; $NaHCO_3$, 0.16; KBr, 0.08; trace elements, 0.082. We obtained 10-fold higher enzyme activity by such optimization. Previously, statistical optimization studies have been carried out using *Moritella* sp.,[17] *Burkholeria* sp.,[16] *Pseudomonas* sp.,[29] and *Geotrichum* sp.[30] Overall, 2.6- to 6.0-fold increases in lipolytic enzyme production were obtained compared to that observed before optimization.

Fed-batch fermentations have been used to obtain high yield of lipase in a fermenter.[31–33] In this study, fed-batch culture performed using optimized media conditions resulted in maximal lipolytic enzyme production of 10.7 U/mL, which was 2 times higher than that of batch cultures with optimized medium. It is difficult to compare the lipolytic activity with other reported lipase activities because of the different substrates and unit definitions used. Using statistical optimization and fed-batch culture, an overall 21-fold increase in lipolytic activity was obtained as compared with unoptimized flask culture conditions.

To date, no practical information is available on the optimization of lipolytic enzyme production by *Psychrobacter* species. This study provides suitable medium and operating conditions for improving lipolytic enzyme production by *Psychrobacter* sp. ArcL13. Consequently, the optimal conditions obtained in the present study would provide a practical basis for large-scale lipase production.

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