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Optimization of Process Parameters for Improved Lipase Production by Hyperthermophilic *Bacillus sonorensis* 4R

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Authors' contributions

This work was carried out in collaboration between all authors. Author HJB designed and made analyses of the study and wrote first draft of the manuscript. Author SZU performed the experimental work and statistical analyses. Author TAK helped to design the study and draft the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To study the effect of process parameters on lipase production of *B. sonorensis* 4R for improved lipase yield.
Place and Duration of Study: School of Life Sciences, SRTM University, Nanded, between Feb 2016 and April 2016.
Methodology: In the present study, the individual and combined effects of process parameters on lipase production by a hyperthermo-alkalophilic strain of *B. sonorensis* 4R are studied. Parameters used in this study were incubation period, temperature, initial pH of medium, carbon and nitrogen sources, substrates and metal salts.
Results: The isolate showed maximum lipase production after 4 days of incubation at 80℃ and pH 8.0 and when growth medium was supplemented with 1% glucose, 1% ammonium sulphate, 100mmol $CaSO_4$ and by using 1% Tween-80 as lipidic substrate. The combined effects of six

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variables (pH, temperature, substrate concentration, carbon source, nitrogen source and metal salt) studied in 12 experimental sets showed highest lipase production (51.33 U/mL) by *B. sonorensis* 4R. In a medium design composed with Tween-80 (1%), $CaSO_4$ (100 mmol), glucose (0.5%), ammonium sulphate (1%), pH (7.5) and when incubated at 80℃, 6.88 fold enhancement over control was observed in lipase production.

Conclusion: In the present study, lipase production from *B. sonorensis* 4R has been optimized using individual and combined effects of simple and easily manageable process parameters. This knowledge will be helpful to many industrial processes to obtain improved enzyme productivity.

Keywords: Hyperthermophilic; alkaline; B. sonorensis 4R; Tween-80; CaSO4; ammonium sulphate.

1. INTRODUCTION

Lipases are the important hydrolytic enzymes having innumerable applications and industrial potential with a projected market of $590.5 million by 2020 [1]. Microbial lipases hold prominent position in industrial market due to their versatile activity, ease of production and purification, convenience for genetic modification, high yield and extracellular nature and their greater stability over plant and animal lipases. Since last few decades, there is a growing interest in lipases, particularly thermostable lipases among the researchers and academia due to their wide scope applications in food, oleochemical, detergents, leather and pharmaceutical industries [2]. Fatty acids released during lipase catalysis are value added products due to their use in surfactants, soap manufacturing, food industry and biomedical applications [3]. Currently, commercial lipases are mainly fungal and bacterial products. Pseudomonas species producing lipase includes *Ps. mendocina, Ps. alcaligenes*, and *Ps. cepacia* [4]. Apart from *Pseudomonas* sp, other bacterial lipases are reported from *Bacillus* sp and *Burkaholderia* sp [5].

Most of these reported lipases are mesophilic in nature, working best at low temperatures. Such enzymes lack or reduce their catalytic efficiency at high temperatures. Since, most of the industrial applications of lipases need harsh conditions of high temperature and alkaline or acidic pH, the performance of mesophilic lipases is often compromised under these conditions. In this view, thermostable lipases isolated from thermophilic organisms are considered as an alternative tool due to their higher thermostability, higher activity at elevated temperature and more resistance to chemical denaturation [6].

Although, some of the thermostable lipases have been reported in last decade, their growing industrial demand intensifies the search of novel thermophilic microorganisms from unexploited ecosystems. In this regard, we have isolated a hyperthermostable alkaline lipase producing strain of *Bacillus sonorensis* 4R from Thar Desert of Rajasthan. The isolation and identification of this Lipolytic strain has been reported earlier [7]. By considering the importance of culture conditions and nutritional parameters in enzyme production by a microbe and to achieve a cost effective enzyme production, the present study was planned to screen various media ingredients (Carbon and nitrogen sources, substrates and metal salts) and cultivation conditions (Temperature, pH and incubation period) so as to obtain optimal thermostable alkaline lipase production.

2. METHODOLOGY

2.1 Lipolytic Organism

B. sonorensis 4R (KT368092) strain was originally isolated from Thar Desert soil sample and identified as a producer of thermophilic alkaline lipase according to Bhosale et al. [7]. The strain was maintained on inorganic salt agar (ISA) medium. For inoculum development, *B. sonorensis* 4R was grown on ISA slants at 50ºC for 48 h. The active culture was inoculated in 25 mL of inorganic salt broth (g/L: K_2HPO_4, 1; $MgSO_4$, 1; NaCl, 1; ammonium sulphate, 2; $CaCO_3$, 2; $FeSO_4$, 0.001;$MnCl_2$, 0.001;$ZnCl_2$, 0.001; tributyrin, 10 mL) and incubated under static conditions. The medium composition and lipase activity titrimetric assay protocol were mentioned previously [7]. In brief, the reaction mixture containing 1 mL of tris-HCl buffer (pH 9.0), 2.5 mL of deionized water, and 3 mL of olive oil emulsion (10% gum arabic emulsified with 5% olive oil), 1 mL of crude enzyme for test and 1 mL of deionized water for blank were added in separate tubes. The reaction mixture was mixed thoroughly by swirling and incubated at 80℃ for 30 min. After incubation, enzyme substrate reaction was terminated by addition of

3 mL of 95% ethanol and mixed by swirling. The amount of fatty acids liberated due to lipase activity was estimated by titrating the contents of assay mixture against 0.05 M NaOH using thymolphthalein as a pH indicator. The end point observed was from colorless to light blue. One unit of lipase was defined as the amount of enzyme required to release 1μmole of fatty acid under assay conditions.

2.2 Optimization of Process Variables for Improved Lipase Production

Various process parameters influencing lipase production by *B. sonorensis* 4R during submerged production at 50℃ temperature and pH 9.0 under static conditions were optimized and the details are given in following sections.

2.2.1 Effect of incubation period

The effect of incubation period on lipolytic potency of the selected strain was studied by inoculating the active culture of *B. sonorensis* 4R in inorganic salt broth of pH 9.0 containing 1% tributyrin as a substrate. The flask was incubated at 50℃ for 9 days. Five ml culture media was removed periodically at an interval of 24 h and used for assessing lipase activity as previously mentioned.

2.2.2 Effect of temperature

To examine the effect of growth temperature on lipase production capacity of the *B. sonorensis* 4R, it was grown separately in different flasks of inorganic salt broth (pH 9.0) containing 1% tributyrin. The flasks were incubated at different temperatures (50℃, 60℃, 70℃, 80℃, 90℃, and 100℃) for 4 days. Enzyme activity was determined after 4 days of incubation. The flask incubated at 50℃ was used as control to compare lipolytic activities at other temperatures.

2.2.3 Effect of initial pH of medium

In order to reveal the effect of initial pH of medium on lipase production ability, the active culture of *B. sonorensis* 4R was inoculated separately in different flasks containing inorganic salt media supplemented with 1% tributyrin. pH's of media were adjusted to different pH (7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5 and 12) with the help of 0.1N NaOH on pH meter. The flasks were incubated at 50℃ for 4 days. Enzyme activity was noted by performing the lipase assay. The flask adjusted at pH 9.0 was used as control flask to determine the effects of other pH's on lipolytic capacity of *B. sonorensis* 4R.

2.2.4 Effect of carbon sources

To study the effect of carbon sources on lipase production by *B. sonorensis* 4R, different carbon sources including glucose, lactose, xylose, sucrose, dextrin, starch, and gum acacia at 1% concentration were added individually in separate flasks containing the inorganic salt media. The broths were incubated at 50℃ for 4 days under static conditions. After incubation, broth was centrifuged and the supernatant was used as crude source of enzyme for lipase assay as mentioned earlier.

2.2.5 Effect of nitrogen sources

For the selection of suitable nitrogen source for lipase production by *B. sonorensis* 4R, different organic and inorganic nitrogen sources such as pyruvate, bile salts, peptone, ammonium sulphate, yeast extract, arginine and soya meal were screened at 1% level. Different flasks of inorganic salt media (pH 9.0) were prepared by supplementing selected sources individually. The active culture of *B. sonorensis* 4R was inoculated in all flasks and the flasks were incubated at 50℃ for 4 days and assayed to determine the enzyme units.

2.2.6 Effect of metal salts

The effect of different metal salts ($CaSO_4$, $MgSO_4$, $ZnCl_2$, at 100 mmol/mL final concentration and $HgCl_2$, $Pb(NO_3)_2$, $CdCl_2$ at 50 mmol/mL final concentration) on lipase production was assessed by inoculating active culture of *B. sonorensis* 4R in different flasks of inorganic salt broth amended with selected metal salts. The flasks were incubated at 50℃ for 4 days under static conditions and used for lipase assay as mentioned before.

2.2.7 Effect of different substrates

Different lipidic substrates such as olive oil, eucalyptus oil, clove oil, castor oil, mustard oil, coconut oil, sesame oil, soya bean oil, Tween-80, wash pump waste water and grease at 1% concentration were supplemented individually in inorganic salt media of pH 9.0 by replacing tributyrin. The flasks were incubated under static conditions at 50℃ for 4 days. After incubation, enzyme activity was determined as mentioned in section 2.1.

Inorganic salt broth supplemented with tributyrin was used as a control medium to reveal the addition effects of carbon and nitrogen sources, metal salts and different lipidic substrates on lipase production.

2.3 Combined Effects of Process Variables on Enhanced Lipase Production by *B. sonorensis* 4R

To evaluate the combined effects [8] of substrate concentration (Tween-80), pH (8.0), temperature (80℃), metals ($CaSO_4$), carbon sources (Glucose) and nitrogen sources (Ammonium sulphate) on production of lipase, 12 experimental sets were conducted as per Table 1 presented in results and discussion.

3. RESULTS AND DISCUSSION

3.1 Optimization of Process Variables for Lipase Production by *Bacillus sonorensis 4R*

3.1.1 Effect of incubation period

Lipase production by *B. sonorensis* 4R started after 24 hr of incubation at 50℃ and increased thereafter with the increase in incubation time. The maximum enzyme activity (7.46 U/mL) was obtained by 4 days of incubation and then decreased gradually from 5th day to 9th day of incubation (Fig. 1). The high incubation time required for enzyme production might be due to the growth of bacterium at high temperature (50℃) under alkaline (pH 9.0) and static conditions. Under these conditions the organism may have high lag period before its exponential growth. The gradual decrease in enzyme production after 4 days might be due to the accumulation of toxic end products of metabolism or exhaustion of available nutrients in medium [8]. Our previous study [8] has showed that *Bacillus* sp gave the maximum yield of (125 U/mL) lipase after 168 h of incubation at 50℃ and pH 9.0 whereas other studies have showed the lipase production was highest after 12 h by *Bacillus* sp RSJ-1 at 50℃ and pH 8.0 with enzyme activity of 10.5 U/mL [9] and 72 h of incubation at 35℃ and pH 8.0 1.2 U/mL by *Bacillus* sp. MPTK 912 [10].

3.1.2 Effect of temperature

Effect of temperature on lipase production by *B. sonorensis* 4R was studied in temperature range of 50-100℃. It was observed that lipase production was enhanced at high temperatures ranging from 6.86 U/mL observed at 60℃ to 27.56 U/mL at 80℃ (Fig. 2). The activities found at 90℃ and 100℃ were higher than those appeared at lower temperatures (50-70℃). The results indicated that the organism is a hyperthermophile having temperature optima of 80℃ [11]. Similar results have been reported for *Thermocrinis ruber* that can tolerate temperature upto 89℃ by Huber et al. [12] while, *T. neapolitana* was reported to have optimum temperature of 77℃ reported by Belkin et al. [13]

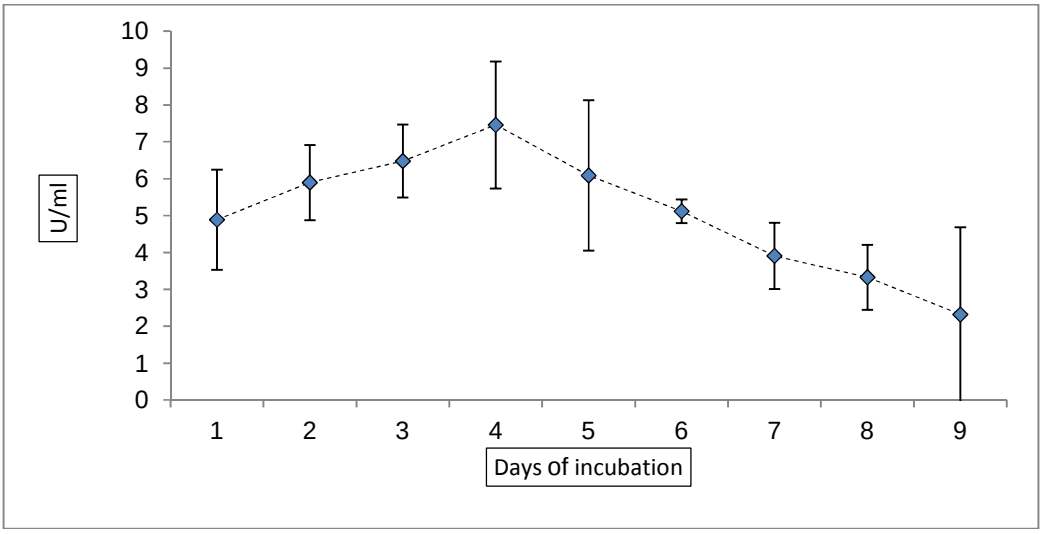

Fig. 1. Effect of incubation period on lipase production by *B. Sonorensis* 4R
Each value represents Mean± S.D (n=3)

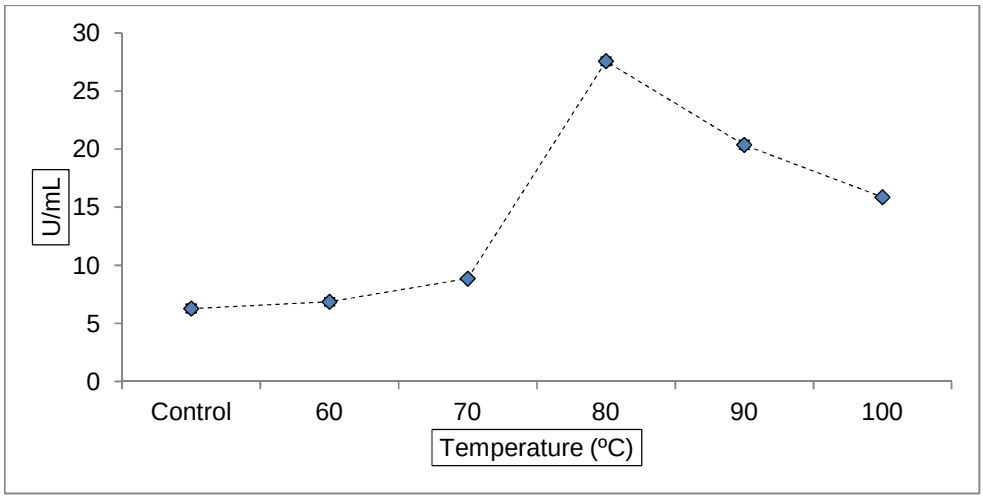

Fig. 2. Effect of temperature on lipase production by *B. sonorensis* 4R
Each value represents Mean± S.D (n=3)

3.1.3 Effect of initial pH of medium

The effects of different pH on lipase production by *B. sonorensis* 4R were observed and the results are summarized in Fig. 3. Lipase production was enhanced at pH 8.0 (14.17 U/mL) as compared to control while it was reduced above and below pH 8.0. Changes in the initial pH of production medium may induce the production of new metabolites that affect the biosynthesis of desired products [8]. Hence, we examined the effect of pH on lipase production by *Bacillus sonorensis* 4R and pH 8.0 was found to be the optimum for enzyme production. Most of the published literature responds pH 8.0 as optimum pH for lipase production in thermophilic *Bacillus* sp [14]. *Bacillus* sp LBN2 was found to be produced optimally at alkaline range pH of 8.0 to 10 [15]. Similar results are also reported for *B. cereus* showing highest production at pH 8.0 (60U/mL) [16]. Increased cultivation of the organism at an unfavorable pH reduces the growth rate of organism and also has marked effects on the level of enzyme production [17] as evidenced by drastically reduced enzyme production at all other pH values.

3.1.4 Effect of carbon sources

Lipase production by thermoalkalophilic *B. sonorensis* 4R was determined at 50℃ and pH 9.0 by measuring the enzyme activity when the strain was growing on different carbon sources. The highest lipase production was

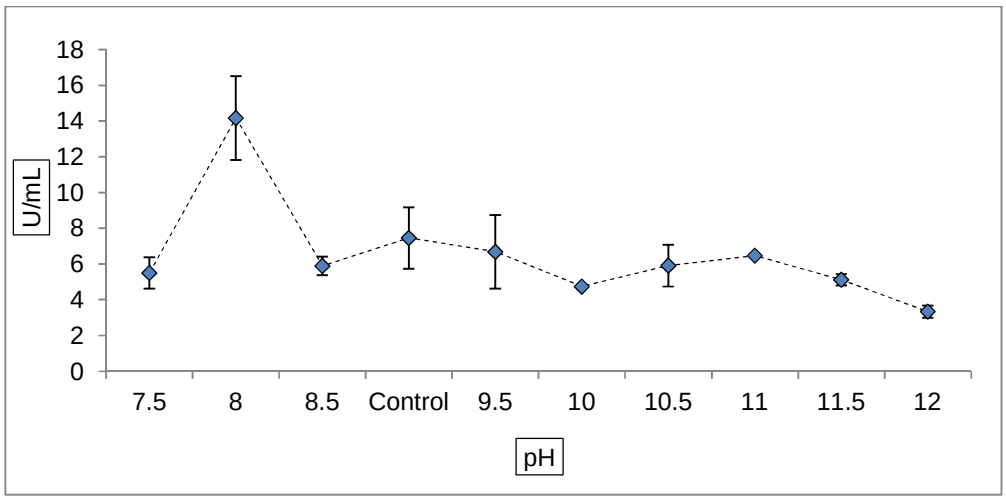

Fig. 3. Effect of initial pH of medium on lipase production by *B. sonorensis* 4R
Each value represents Mean± S.D (n=3)

obtained with 1% glucose (17.65 U/mL) followed by sucrose (3.94 U/mL) and xylose (3.34 U/mL). In presence of dextrin, the enzyme activity showed by *B. sonorensis* was lower than control whereas the activity was negligible or absent when starch, gum acacia and lactose were incorporated in the medium (Fig. 4). Glucose can stimulate lipase activity as it supported the growth of thermoalkalophilic *B. sonorensis* 4R. Ability of an organism to drive a metabolic reaction and grow in presence of specific carbon source is dependent on typical enzymatic machinery present in the cell. In the present study, the strain 4R grew well in presence of glucose. However, lowest enzyme units recorded in the presence of lactose and activity was completely inhibited in presence of starch and gum acacia indicating that these are not suitable for lipase production. Utilization of glucose as carbon sources by *Bacillus* sp was also reported [9,18] in previous literature. Among other carbon sources, consumption of fructose, maltose, lactose and salicin is observed by Kumar et al. [10] and Hasan et al. [19] respectively.

3.1.5 Effect of nitrogen sources

The addition effect of different nitrogen sources on enzyme production by *B. sonorensis* 4R is shown in Fig. 5. The lipase production by 4R was enhanced markedly when 1% ammonium sulphate was amended in the medium (21.08 U/mL) in comparison to arginine (7.45 U/mL) and yeast extract (7.27 U/mL) supplementation. Lipase production was not noticeable in presence of peptone, pyruvate and soya meal and was completely inhibited in presence of bile salts. Ammonium sulphate is highly soluble in water. Hence, readily available nitrogen source for microbial growth. Further, its high ionic strength releases ammonium ions in aqueous media that can be easily incorporated as part of different amino acids and hence plays important role in protein synthesis process [17]. This might be the reason why ammonium sulphate supported well for lipase production by 4R.

3.1.6 Effect of metal salts

The effect of different metal salts added to the production medium on lipase production is presented in Fig. 6. Salts of calcium ions ($CaSO_4$) highly stimulated (16 U/mL) the lipase production by *Bacillus sonorensis* 4R whereas, enzyme production was reduced when $ZnCl_2$ (3.4 U/mL) and $MgSO_4$ (5.13 U/mL) were supplemented in the medium. Complete inhibition of enzyme activity was observed when media was supplemented with $HgCl_2$, $Pb(NO_3)_2$ and $CdCl_2$. Optimal effects of Ca^{++} supplementation on thermostable lipase production were previously documented by Bora and Kalita [20], Salameh and Weigel [21], Nath and Hindumathy [22] whereas Anandhi et al. [23] observed enhancement in lipase production at 0.06 g 50 mL^{-1} concentration of $MgSO_4$. Calcium ions play essential roles for many microbial species. In addition to being a binder and stabilizer of lipase enzyme, they are also important in maintaining cell wall rigidity, stabilizing oligomeric proteins and covalently bounding protein peptidoglycan complexes in the outer membrane [24]. Hence, we can expect stimulated lipase production in presence of calcium ions.

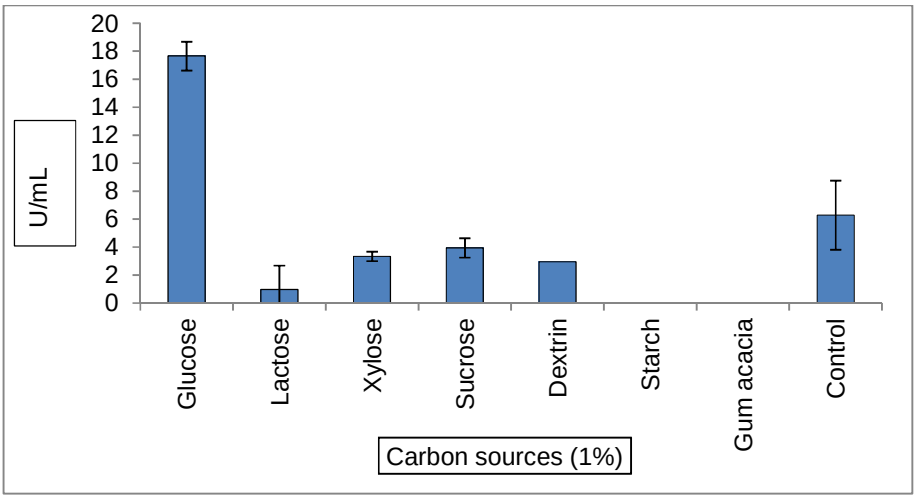

Fig. 4. Effect of carbon sources on lipase production by *B. sonorensis* 4R
Each value represents Mean± S.D (n=3)

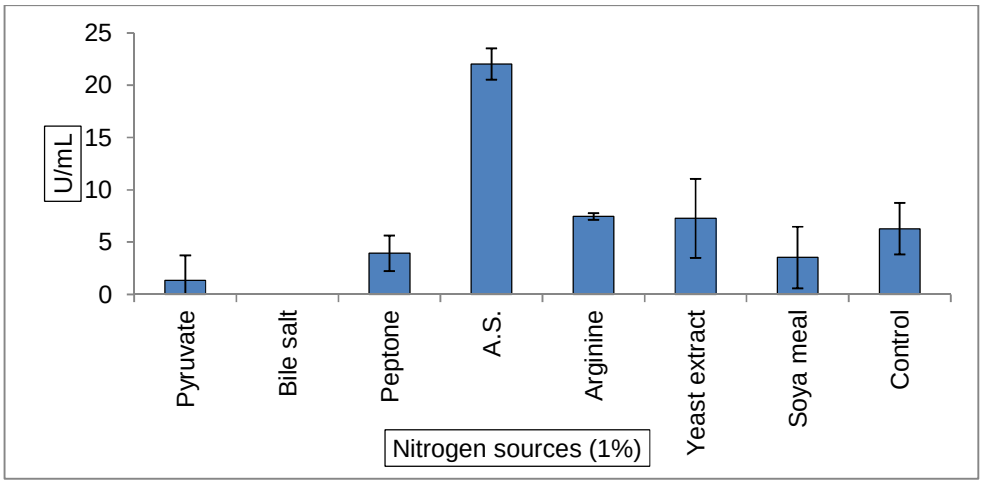

Fig. 5. Effect of nitrogen sources on lipase production by *B. sonorensis* 4R
Each value represents Mean± S.D (n=3)

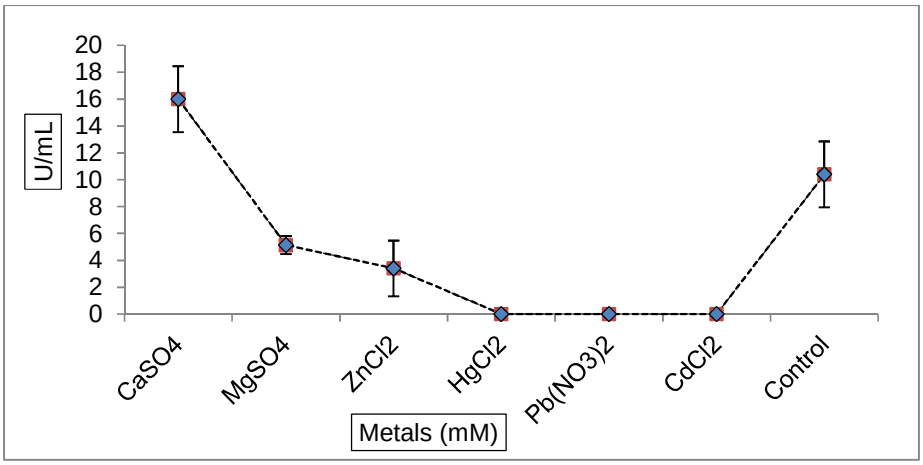

Fig. 6. Effect of metal salts on lipase production by *B. sonorensis* 4R
Each value represents Mean± S.D (n=3)

3.1.7 Effect of different substrates

The enzyme production was highest when Tween-80 was used in production media as compared to olive oil (6.49 U/mL), eucalyptus oil (10.4 U/mL), castor oil (7.46 U/mL), clove oil (7.37 U/mL), mustard oil (4.02 U/mL), coconut oil (8.24 U/mL), sesame oil (7.98 U/mL), soya bean oil (8.24 U/mL) and tributyrin (5.29 U/mL) which was used as a control and also by cheap substrates like waste water from wash pump (8.24 U/mL) and grease (5.32 U/mL) as indicated in Fig. 7. Supplementation of different lipidic substrates in production medium greatly affected the lipase production capacity of *B. sonorensis* 4R. Lipase is an extracellular inducible enzyme whose production is only detectable in presence of suitable substrate. Tween-80 acts as a structural analog of lipids and appears in the form of esterified lipid. Hence, it is considered as good substrate for lipase production. Sharma et al. [9] also reported lipase production using Tween-80 as a substrate from thermophilic *Bacillus* sp. RSJ-1.

3.2 Combined Effects of Process Variables on Lipase Production by *B. sonorensis* 4R

The combined effects of selected parameters were studied in 12 experimental sets and the respective lipase units were presented in Table 1. Highest enzyme units were observed

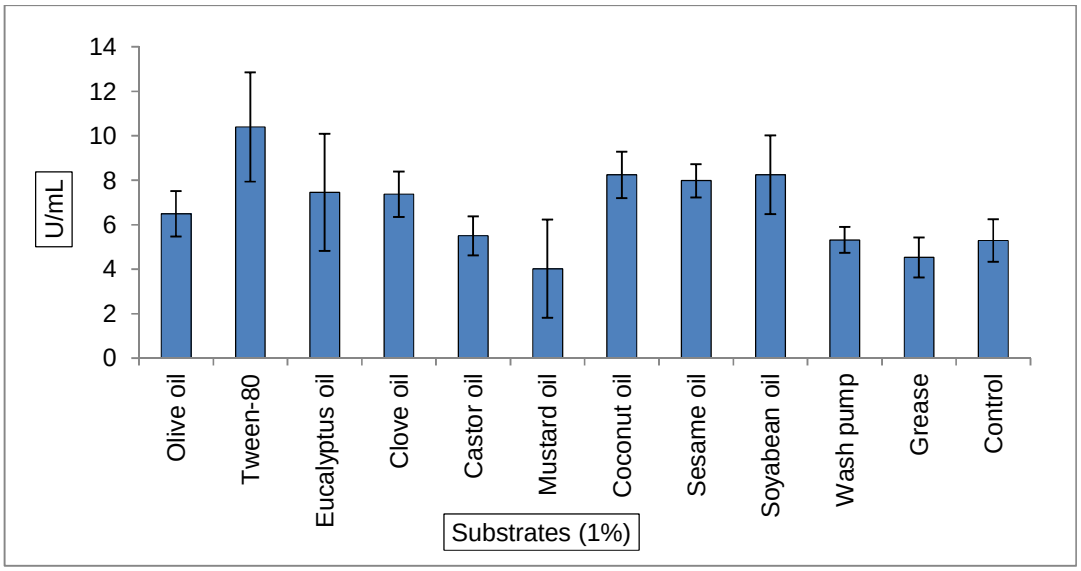

Fig. 7. Effect of substrates on lipase production by *B. sonorensis* 4R
Each value represents Mean± S.D (n=3)

Table 1. Optimization of lipase production

Sets	Components						U/mL	Mean±S.D.
	pH	Temperature (ºC)	Tween-80 (%)	$CaSO_4$ (mmol)	Glucose (%)	A.S. (%)		
Control	9.0	50	-	-	-	-	7.46	0.26
1	8.0	80	1	100	1	1	44.25	0.25
2	8.0	80	0.5	50	0.5	1	40.71	0.35
3	8.0	70	1	100	0.5	0.5	26.55	0.52
4	8.0	70	0.5	50	1	1	41.31	0.51
5	7.5	80	1	50	1	0.5	35.40	0.40
6	7.5	80	0.5	100	0.5	1	45.45	0.45
7	7.5	70	0.5	100	1	0.5	21.24	0.36
8	7.5	70	1	50	0.5	1	20.64	0.52
9	8.0	70	1	50	1	0.5	15.93	0.55
10	8.0	80	1	50	0.5	0.5	23.61	0.45
11	7.5	80	1	100	0.5	1	51.33	0.16
12	7.5	70	1	100	1	1	36.00	0.45

Note: Control flask contains inorganic salt broth supplemented with 1% tributyrin as a substrate

during 11th experimental set representing 51.33 U/mL yield. The composition of optimal medium designed as per 11th experimental set is also highlighted in Table 1. When compared to other experimental designs (15.39-44.25 U/mL) and control set (7.46 U/mL), the selected optimal glucose tween Inorganic salt broth medium showed 6.88 fold enhancement in lipase production over control medium used for lipase production by *B. sonorensis* 4R.

4. CONCLUSION

In the present study, a hyperthermophilic alkaline lipase producer strain of *B. sonorensis* 4R has been optimized for improved lipase production. This study first time demonstrated the use *B. sonorensis* 4R strain for the production optimization of lipase at 80℃ temperature and pH 8.0. The high temperature tolerance of this lipase will make it an enzyme of industrial choice

and considered to be a potential candidate for its successful commercialization.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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