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PRODUCTION, CHARACTERIZATION AND APPLICATION OF LIPASES FROM ENDOPHYTIC AND RHIZOSPHERIC BACTERIAL ISOLATES

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Abstract

Bacterial lipases are diverse and are widely used as industrial enzymes. The widespread application of bacterial lipases has encouraged researchers to explore new lipase-producing bacterial strains. In this study, two bacterial strains associated with the salt marsh plant, *Arthrocnemum macrostachyum* were investigated for lipase production. The endophytic *Bacillus licheniformis* E3 and rhizospheric *Bacillus* sp. R7 produced 4.37 and 16.67 IU mL^{-1} of lipase, respectively, when cultivated in the medium containing the powdered seeds of *A. macrostachyum*. The design of the experiment using the Plackett-Burman design helped improve the titers by 1.2 to 2 folds. Moreover, the lipase activity of both strains was characterized using the Central Composite design. Under optimum conditions, lipase activity reached to 100-200 IU mL^{-1} which corroborated the 16-32 folds enhancement in lipase activity. Both lipases were compatible with the commercial detergent and could remove the olive-oil stain from the cotton fabric.

Key words: *Bacillus licheniformis*; Central Composite design; Halophytes; Plackett Burman design.

Introduction

Lipases are industrially important biocatalysts isolated from all organisms, including animals, plants, bacteria, molds, and yeasts (Baruah *et al.,* 2018). Indeed, considering the enzyme market share, lipases belong to the most important group of enzymes along with proteases and carbohydrases. Owing to their convenient large-scale cultivation, availability of genetic manipulation techniques, and adaptation to a variety of environmental stresses, bacteria are the most important source of lipases (Yao *et al.,* 2021). Bacterial lipases (EC 3.1.1.3) are generally characterized as α/β-hydrolases with a catalytic triad of serine, histidine, and aspartic acid. Catalytic action of lipases degrades triglycerides into diglycerides, monoglycerides, glycerol, and fatty acids. However, their broad substrate specificity and regio- and stereospecificity enable lipases to exhibit different actions, including esterification, transesterification, and deacetylation. Thus, lipases have applications in a variety of chemical synthesis and bioremediation processes. Among bacteria, members of the genus *Bacillus* have always been referred to as houses of versatile metabolites with industrially relevant properties. *B. licheniformis* is a Gram-positive rod that has remained an industrially important microorganism for several decades and has been reported for amylase, penicillinase, protease, pentosanase, pectinase and lipase production in good yield. In an early report, *B. licheniformis* was described for its lipolytic potential (Khyami-Horani, 1996), with remarkable stability over a wide range of temperatures under alkaline conditions. Another strain of *B. licheniformis*, NCU CS-5, was studied for its stability in a variety of environments and was reported to have the potential to degrade 2,4-D butyl esters (Zhao *et al.,* 2021). Malekabadi *et al*., (2018) reported about the lipase production from *B. licheniformis* KM12 and emphasized over the stability of the enzyme in presence of various organic solvents. Likewise, a strain of *B. licheniformis* isolated from desert soil exhibited a promising ability to produce lipase with industrially relevant properties (Verma *et al.,* 2020). Pandey *et al*., (2015) isolated various lipolytic strains of *B. licheniformis* and one strain of *B. tequilensis* from a hot spring. *B. tequilensis* has also been isolated from the medicinal plant *Angelica dahurica*, and its potential as a biological control agent has been investigated (Li *et al.,* 2018). Kang *et al*., (2019) demonstrated the ameliorative effect of a rhizospheric strain of *B. tequilensis* SSB0 on soybean plant growth. Indeed, endophytic microbes manifest a variety of activities that provide various benefits to plants. However, lipase production by bacterial strains associated with coastal plants has not been frequently reported.

Colla *et al*., (2015) after screening different oils concluded that olive oil is a suitable substrate for the production of lipase. Several studies have also elaborated on olive oil as an optimal substrate for lipase production (Kok *et al.,* 1996). However, the search for alternative cost-effective substrates remains in demand. In this context, biomass and seeds from halophytic plants provide prospects for their utilization as raw fermentation materials. *Arthrocnemum macrostachyum*, a halophyte and a member of the family Chinopodiaceae, is a shrub found in the coastal region of Mediterranean basins and South Asia (Lopes *et al.,* 2016). Along with their ability to tolerate high salt concentrations, plants have also been reported to contain nutrients, carotenoids, vitamin C, and phenolics. It produces large numbers of brown and black seeds. Its seeds germinate in highly saline soils (Gul & Khan, 2001). In addition, the presence of high quantities of polyunsaturated fatty acids has also been reported in this plant *(*Lopes *et al.,* 2016); however, these studies mainly relied on the medicinal impact of the constituents instead

of considering the seeds for cost-effective lipase production. Indeed, the seeds contain as much as 25% oil, primarily α-linolenic and linoleic acids (Gul & Khan, 2001). This study describes the use of *A. macrostachyum* in two ways. First, lipolytic bacterial strains were isolated from the tissues of this plant or its associated rhizospheric soils, and second, by using its seeds as a source of lipids for the production of lipase. The role of endophytes as plant growth-promoting organisms has been known for many years; however, interest in exploring endophytic strains and their biotechnological applications is growing. The endophytic community of *A. macrostachyum* has been described in detail based on genetic diversity (Mora-Ruiz *et al.,* 2016). The plant is known for its tolerance to metals, and its role in phytoremediation has been established (Kang *et al.,* 2019). Initial reports in this context described the role of *A. macrostachyum* endophytes in remediation of metal-ridden soils (Del Mora-Ruiz *et al.,* 2018) without considering the enzymatic potential of those organisms. A recent report on endophytic isolates of *A. macrostachyum* described their potential for industrially important enzymes (Khan *et al.,* 2022); however, to best of our knowledge, the plant or its seeds have not been reported as a growth medium for the production of lipase. Therefore, this study is designed to screen endophytic and rhizospheric bacteria associated with *A. macrostachyum* for lipase production using different oils and powdered seeds of *A. macrostachyum.* Production of lipase was optimized by Plackett Burman design and produced lipase was used to remove oil stain from the cotton fabric.

Material and Methods

Bacterial strains, media and chemicals: The bacterial strain, B. licheniformis E3 (GenBank Accession No. ON060355) and Bacillus sp. R7 (GenBank Accession No. ON060361) were originally isolated from the coastal plant, A. macrostachyum and its associated rhizospheric soil (Khan *et al*., 2022). Cultures were revived from glycerol stocks on Nutrient agar and maintained at 4ºC. For lipase production, inoculum was prepared by cultivating an isolated colony of the strains in nutrient broth and was added by keeping 0.3 OD_{600} in the production medium. Mineral salt medium (MSM) comprised of g L^{-1} of NH_4NO_3 0.0005, KH2PO4 0.0001, K2HPO4 0.0002, $MgSO_4.7H_2O$ 0.00005, KCl 0.000001, $CaCl_2$ $1x10^{-6}$, $FeSO_4.7H_2O$ $2x10^{-6}$ containing a suitable carbon source was used as a production medium. All chemicals were purchased from Sigma Aldrich (USA) unless specified.

Lipase production using oils and plant seeds: Production medium was comprised of MSM supplemented with 0.01 g L^{-1} glucose and added with 0.05 g L^{-1} of either an oil (olive oil, mustard oil, castor oil or coconut oil) or powdered seeds (of *Panicum antidotale*, *Phragmites karka*, *Urochondra setulosa*, *Typha domingensis*, *A. macrostachyum* and *Zaleya pentandra*). Inocula of the bacterial strains E3 and R7 were separately added and cultivated at 32ºC for 48 h. Cell-free culture supernatant (CFCS) was obtained by centrifugation at 5000 *xg* for 15 min and was taken as lipase preparations.

Lipase assay: Cell free extract of produced Lipase or CFCS (50 µL) was added to 200 µL of 0.1 mM p-nitrophenyl palmitate (pNPP) and the mixture was incubated at 30°C for 10 min. The reaction was stopped by adding 20 µL of NaOH (10% w/v) and absorbance at 405 nm was noted. Standard solutions of p-nitrophenol were prepared to calculate lipase units.

Lipase production using *A. macrostachyum* seeds as substrate: Since both strains produced higher titers of lipase during their cultivation on *A. mascrostachyum* seeds, factors affecting lipase production using these seeds were investigated by employing the Plackett Burman design (PBD). Seven independent factors (pH, temperature, inoculum size, concentration of *A. macrostachyum* seeds, carbon and nitrogen sources and incubation time) were studied for *B. licheniformis* E3 and *Bacillus* sp. R7. In preliminary studies, strain E3 yielded comparable titers in the presence of glucose or glycerol, therefore, these two carbon sources were included in the PBD for this strain. Although the strain R7 could not produce lipase upon cultivation on glycerol, variable concentrations of glucose were included in the PBD for this strain. Minitab18 software was used to design the matrix that suggested 12 different experimental runs. Typical cultivation for PBD was carried out by transferring 5% or 10% of bacterial inoculum to 0.1 or 0.2% of powdered seeds of *A. macrostachyum* supplemented with 1% ammonium nitrate or yeast extract and glucose or glycerol with a pH adjusted to 3.5 or 9 and incubated at 28 or 32ºC for 24 to 48 h. Post-incubation CFCS was assayed for lipase activity and IU mL^{-1} of lipase was considered as a response to analyze the PBD.

Effect of nutrient broth and mineral salt media on lipase production: Induction and repression of lipase production was performed by inoculating *B. licheniformis* E3 and *Bacillus* sp. R7 into nutrient broth and MSM separately. After 24 h of incubation, media was centrifuged in order to collect the cells. Cells were washed 3 times with 0.5 mL of distilled water and inoculated into 5 mL nutrient broth and MSM. After incubation, media was centrifuged, and the enzyme assay was performed by using cell free culture supernatant (CFCS).

Characterization of lipase: The Central Composite design (CCD) in Response Surface methodology was adopted to optimize various factors affecting lipase activity from strain E3 and R7. Four variables including temperature, pH, reaction time and substrate amount were considered. The variables were adjusted to be high (+1) and low (-1) with α= 0.5. The design was adjusted with 5 levels. Twenty-five experiments were suggested by Minitab software. The lipase assay was performed and IU mL^{-1} was taken as response.

Application of lipase to remove olive oil stains: Cotton fabric (10 × 10 cm^2) was taken for soiling and olive oil was spot inoculated onto the fabric and air dried at room temperature. The setup was prepared by washing and soaking olive oil-soiled cotton fabric with i) Lipase from E3 in a buffer of pH 9 ii) Lipase from E3 with detergent iii) Lipase from R7 in a buffer of pH 9 iv) Lipase from R7 with

detergent and v) unheated detergent. The washing was carried out at 30°C in a shaker at 100 rpm for 30 min. The fabric was rinsed three times with distilled water, air-dried and images were recorded.

Statistical analysis: All experiments were conducted in triplicate and the mean values are presented here. Minitab version 18 was used for statistical analysis.

Results

The isolates were cultivated in MSM containing either a commercially available oil or seeds of halophytic plants. The strain E3 produced more titers (Table 1) in presence of oil as substrate (0.21-8.75 IU mL^{-1}) compared to the halophytic seeds (0-4.37 IU mL^{-1}). While the rhizospheric isolate, R7, yielded much higher titers in presence of any of the substrate tested (4.87-20.65 IU mL^{-1}). Indeed, the cultivation of R7 strain in the medium containing halophytic plants' powdered seeds yielded the lipase titers in the range of 4-16 IU mL^{-1} that were comparable to the lipase production on commercially purified oils. Since, both the strains produced higher titers of lipase (4.37 and 16.69 IU mL^{-1}) in presence of the powdered seeds of *A. macrostachyum* and considering the cost benefits of halophytic plant's powdered seeds, this substrate was used in further experiments.

Studies on lipase production by R3 and E7: It was observed that experimental run no. 02 of Plackett Burman design (PBD) in which MSM amended with 0.5% glycerol and yeast extract containing 0.1% w/v *A. macrostachyum* powdered seeds with pH adjusted to 9 was used to cultivate 5% inoculum of B. licheniformis E3 at 32°C for 24 h, the strain yielded the highest lipase titers of 8.24 IU mL^{-1} (Table 2). Whereas Bacillus sp. R7 showed the highest lipase production of 18.75 IU mL^{-1} (Table 3) under the conditions of the experimental run no 05 (temperature 32°C, incubation time 48 h, media containing 0.5% glucose and yeast extract, with 5% inoculum and 0.2% *A. macrostachyum* powdered seeds with pH adjusted to 9).

A response analysis was carried out after experimental runs as proposed by the PBD. The pareto charts illustrated that none of the factors appeared as significant for affecting the lipase production by *B. licheniformis* E3 (Fig. 1A) and *Bacillus* sp. R7 (Fig. 1B). The R-sq values of E3 and R7 were found as 66.07% and 51.59%, respectively which demonstrated that both the designs were not significant.

Characterization of lipase activity

Lipase activity by *B. licheniformis* sp. E3: Likewise, to characterize the lipase activity from the strain E3, another design was executed, where the data showed variation from 0.831 to 140.39 IU mL^{-1} in 25 experimental runs (Table 4). The result of the experimental run order 6 found to be the most encouraging with 140.39 IU mL^{-1} when the lipase assay was conducted for just a minute at 45°C and pH 5, with the substrate volume 200 µL. The titers indicate about >32 folds increase than the initial titers under un-optimized conditions. While in the run order 15 the activity was very low (0.831 IU mL^{-1}) when assay conditions included temperature, 25°C, pH 9, reaction time 20 min and the substrate volume 50 µL.

The data generated through CCD was analyzed by taking the activity (IU mL^{-1}) as response. The R squared value of 87.22% indicated about the reliability of the model affirmed. The software also generated Pareto chart (Fig. 2). However, only the reaction time was found to be a significant factor.

Table 1. Quantification of lipase production by using different substrates.

Isolates	Lipase production (IU mL^{-1})*									
		Substrates								
	OO	MO	CO	CSO	PA	PK	US	TD	AM	ZP
E3	4.11	8.75[a]	8.34	0.21[a]	0	3.21	0	0	4.37	2.82[a]
R7	12.78	13.79	20.65[a]	5.55	4.83	13.95	13.72[a]	14.16	16.69	8.89

The isolates were cultivated in the medium containing Olive oil (OO), Mustard Oil (MO), Coconut oil (CO), Castor oil (CSO), *Panicum antidotale* (PA), *Phragmites karna* (PK), *Urochondra setulose* (US), *Typha domingensis* (TD), *Arthrocnemum macrostachyum* (AM) and *Zaleya pentandra* (ZP); *Insignificant standard deviation

Table 2. Plackett-Burman design for the screening of significant factors influencing lipase production by *B. licheniformis* E3.

Run order	Temperature (°C)	Nitrogen source (0.1%)	Carbon source (0.5%)	pH	AM seeds concentration (%)	Inoculum size (%)	Incubation time (h)	Lipase production (IU mL^{-1})*
1	32	Ammonium nitrate	Glucose	3.5	0.1	5	48	2.8
2	32	Yeast extract	Glycerol	9	0.1	5	24	8.24
3	28	Yeast extract	Glucose	3.5	0.2	5	24	6.95
4	32	Ammonium nitrate	Glucose	9	0.1	10	24	5.61
5	32	Yeast extract	Glycerol	9	0.2	5	48	4.98
6	32	Yeast extract	Glucose	3.5	0.2	10	24	5.15
7	28	Yeast extract	Glucose	9	0.1	10	48	2.69
8	28	Ammonium nitrate	Glucose	9	0.2	5	48	7.12
9	28	Ammonium nitrate	Glycerol	9	0.2	10	24	5.90
10	32	Ammonium nitrate	Glycerol	3.5	0.2	10	48	4.02
11	28	Yeast extract	Glycerol	3.5	0.1	10	48	3.01
12	28	Ammonium nitrate	Glycerol	3.5	0.1	5	24	1.95

Seven factors were selected including pH, temperature, inoculum size, *A. macrostachyum* (AM) seeds concentration, carbon source concentration, nitrogen source and incubation time; *Insignificant standard deviation

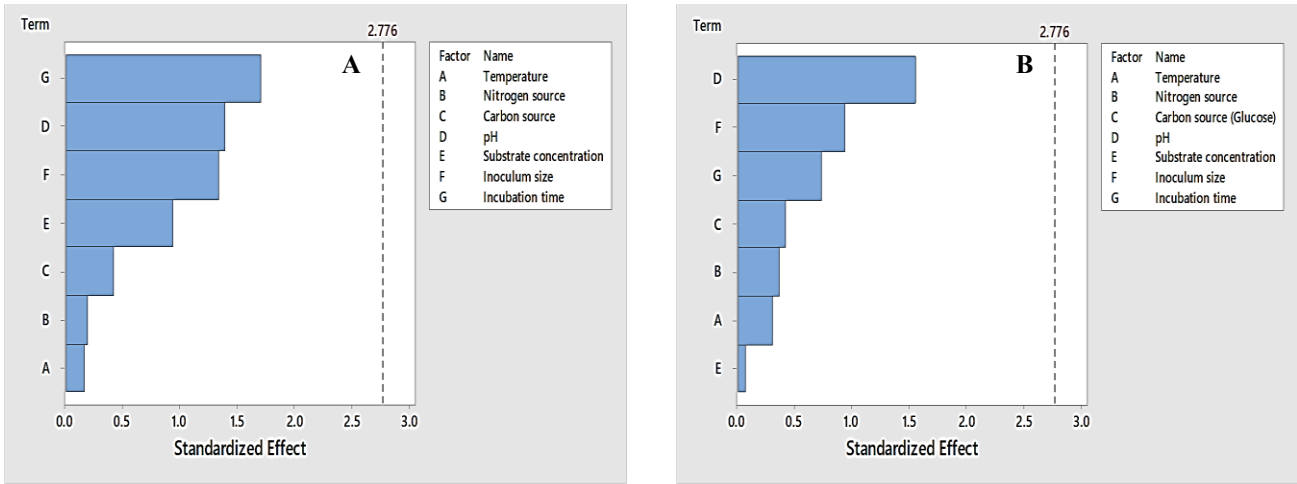

Fig. 1. Pareto charts showing effect of pH, temperature, inoculum size, *A. macrostachyum* seeds concentration, carbon source and concentration, nitrogen source and incubation time on lipase production by (A) *B. licheniformis* E3 (B) *B. tequilensis* R7.

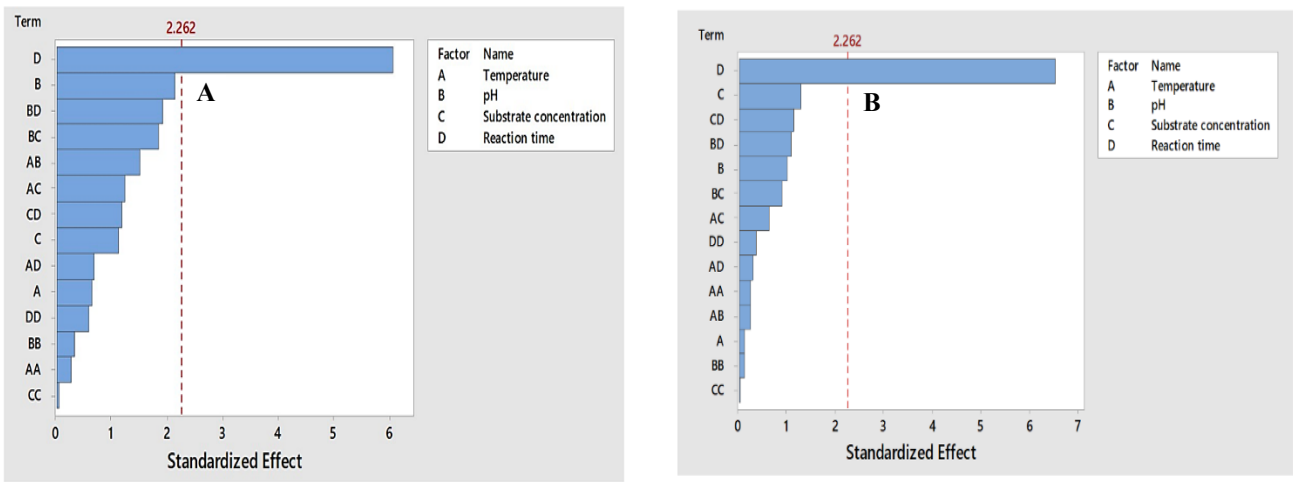

Fig. 2. Pareto Chart of the Standardized effect. Response is the IU mL^{-1} of Lipase activity by (A) *Bacillus licheniformis* E3 (B) *Bacillus* sp. R7.

Interaction between factors regarding their impact on the lipase activity was investigated by generating the contour plots (Fig. 3). Temperature and pH had inverse relationship and higher lipase activity can be achieved by keeping the temperature high but at low pH. Whereas, the amount of substrate and temperature had a direct relationship as keeping both the variables at higher side resulted in the higher lipase activity. The contour plot exhibited that the reaction time should be kept shorter if the reaction is carried out at low temperature. Similarly, a shorter reaction time is optimal if the reaction had to be conducted at low pH. But at the enzyme reaction at low pH, higher substrate amount is required.

The optimized condition for the enzyme activity was also generated and the software gave a single experimental run with the expected lipase activity of 126.78 IU mL^{-1} under optimum conditions of 45ºC, pH 5 substrate volume 200µL and the reaction time of 1min. The obtained value of 124.93 IU mL^{-1} showed that the model accurately predicted the experimental conditions.

Lipase activity by *Bacillus* sp. R7: Central Composite design (CCD) was used to optimize the assay conditions for lipase activity produced by *Bacillus* sp. R7. After performing the lipase assays under suggested conditions by the CCD, it was found that the activity of the enzyme varied greatly from 3 to 353 IU mL^{-1} (Table 4) reflecting the pronounced effect of assay conditions. The result of the experimental run order 6 found to be the most promising with 353.09 IU mL^{-1} when the lipase assay was conducted for just one minute at 45ºC and pH 5, with the substrate volume 200 µL (corresponded to 1µM amount). Whereas, the experimental run order 9 gave the lowest activity of 3.31 IU mL^{-1} under the conditions of pH 5, 25ºC with 50 µL substrate volume and reaction time of 20 min.

CCD was analyzed by taking the IU mL^{-1} by the strain R7 as response. The R squared value of 87.15% indicated about validity of the model. In Pareto chart, only the reaction time was found as significant (Fig. 2). The effects of variables (pH, reaction time, and temperature and substrate volume) and their effect on the lipase activity by *Bacillus* sp. R7 were described in the form of contour plots (Fig. 4). The plots were obtained by plotting the response (IU mL^{-1}) against the two variables. The graph presented that at high temperature and low pH (5), the enzyme exhibited higher activity. While the temperature and amount of substrate interacted in a way that the activity can be increased by keeping both the variables towards higher side. Whereas, temperature had a different interaction with the reaction time as at higher temperature, shorter duration

is needed for the reaction. pH and the amount of the substrate had a negative interaction as keeping pH towards lower side required a higher volume of the substrate.

The software further provided optimized conditions and presented a single experimental run with the predicted lipase activity. Under optimum conditions of temperature 45°C, pH 5, substrate volume of 200 mL and reaction time of 1 min, a titer of 271.58 IU mL^{-1} was predicted. After performing lipase assay an activity of 268.67 IU mL^{-1} was obtained indicating about fitness of the model. This activity corresponded to 16 folds increase in the lipase titers compared to the activity under initial un-optimized conditions.

Application of lipase as a detergent additive: Lipases from the strains E3 and R7 were separately investigated for their activity as detergent additives. A piece of cotton fabric was soiled with olive oil and washed with detergent supplemented with lipases and the results were compared with the appropriate controls including an unstained cotton fabric and an olive oil soiled cotton fabric (Fig. 5). The data showed that the crude lipase preparation from both the strains, *B. licheniformis* E3 and *Bacillus* sp. R7, in a buffer of pH 9 or in detergent effectively cleared the stain of olive oil from the cotton fabric (Fig. 5).

Table 3. Plackett-Burman design for the screening of significant factors influencing lipase production by *Bacillus* sp. R7.

Run Order	Temperature (°C)	Nitrogen source (0.5%)	Glucose (%)	pH	AM seeds concentration (%)	Inoculum size (%)	Incubation time (h)	Lipase production (IU mL^{-1})*
1	32	Ammonium nitrate	1	3.5	0.1	5	48	5.78
2	32	Yeast extract	0.5	9	0.1	5	24	6.93
3	28	Yeast extract	1	3.5	0.2	5	24	5.74
4	32	Ammonium nitrate	1	9	0.1	10	24	13.46
5	32	Yeast extract	0.5	9	0.2	5	48	18.75
6	32	Yeast extract	1	3.5	0.2	10	24	3.47
7	28	Yeast extract	1	9	0.1	10	48	14.98
8	28	Ammonium nitrate	1	9	0.2	5	48	12.70
9	28	Ammonium nitrate	0.5	9	0.2	10	24	5.67
10	32	Ammonium nitrate	0.5	3.5	0.2	10	48	6.54
11	28	Yeast extract	0.5	3.5	0.1	10	48	5.16
12	28	Ammonium nitrate	0.5	3.5	0.1	5	24	12.86

Seven factors were selected including pH, temperature, inoculum size, *A. macrostachyum* (AM) seeds concentration, carbon source concentration, nitrogen source and incubation time; *Insignificant standard deviation

Table 4. Central Composite design to optimize lipase activity from *B. licheniformis* E3 and *Bacillus* sp. R7.

Run order	Factors				Lipase activity (IU mL^{-1})*	
	Temperature (°C)	pH	Substrate concentration (mM)	Reaction time (min)	*B. licheniformis* E3	*Bacillus* sp. R7
1	25	5	50	1	54.48	220.12
2	45	5	50	1	64.66	97.98
3	25	9	50	1	102.27	118.20
4	45	9	50	1	7.24	183.77
5	25	5	200	1	113.84	197.633
6	45	5	200	1	140.39	353.086
7	25	9	200	1	41.87	195.36
8	45	9	200	1	37.51	139.096
9	25	5	50	20	5.64	3.31
10	45	5	50	20	2.23	8.176
11	25	9	50	20	1.00	9.137
12	45	9	50	20	1.01	3.971
13	25	5	200	20	2.66	8.7961
14	45	5	200	20	4.82	8.47
15	25	9	200	20	0.83	21.79
16	45	9	200	20	0.92	1.981
17	35	7	125	10.5	8.77	26.435
18	30	7	125	10.5	13.90	19.4334
19	40	7	125	10.5	23.39	28.286
20	35	6	125	10.5	10.22	10.615
21	35	8	125	10.5	10.54	12.046
22	35	7	87.5	10.5	16.18	12.4522
23	35	7	162.5	10.5	12.37	20.86
24	35	7	125	5.75	33.54	38.618
25	35	7	125	15.25	12.54	15.7121

*Insignificant standard deviation

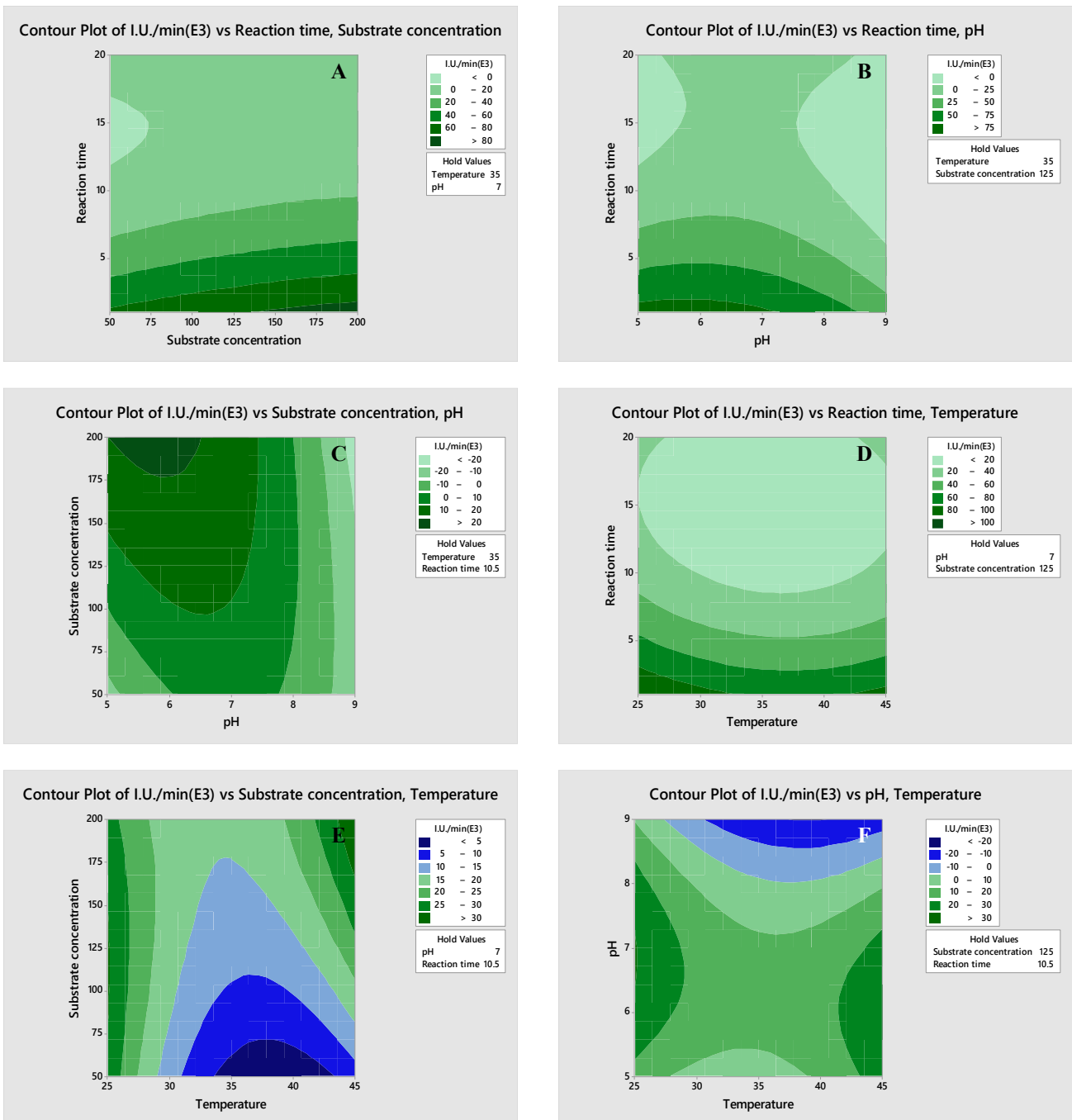

Fig. 3. Contour plots representing interactive effects of variable on the lipase activity by *B. licheniformis* E3.

Discussion

Lipases are one of the most important industrial biocatalysts with diversified applications. In the detergent industry, lipases from *Pseudomonas fluorescens*, *Serratia arlettae*, *B. cepacia*, *Candida* sp. (Phuah *et al.*, 2015), *Geobacillus* sp., *B. licheniformis*, *B. flexus*, *S. marcescens*, and *B. pumilus* (Niyonzima & More, 2015) are used. However, the new and novel strains are routinely investigated to obtain lipases in better yield and with compatible properties for the industrial applications. Here, an endophytic strain and a rhizospheric strain isolated from a coastal plant, *A. macrostachyum*, were studied for lipase production and their lipases were characterized for the optimal activity. Amongst different oils and plant's powdered seeds tested, both the bacterial strains produced higher titers of lipases when cultivated on the seeds of *A. macrostachyum* indicating about the natural selection of the isolate for this substrate. Previously, endophytic actinomycetes isolated from different medicinal plants were screened for lipase production and out of 69, eight isolates were found as lipolytic (Lestari *et al.*, 2018). This indicates that lipolytic activity is not widely distributed amongst endophytes, yet the exploration of new strains as lipase source is an ongoing endeavour. Recently, Silva *et al.*, (2024) used residual palm oil, residual soybean oil, palm oil, soybean oil and olive oil for lipase production by *Penicillium roqueforti* ATCC 10110, however, these oils have many other applications. In this study, the bacterial strains isolated from *A. macrostachyum* were cultivated in presence of the powdered seeds of the same plant and the lipase production was noted.

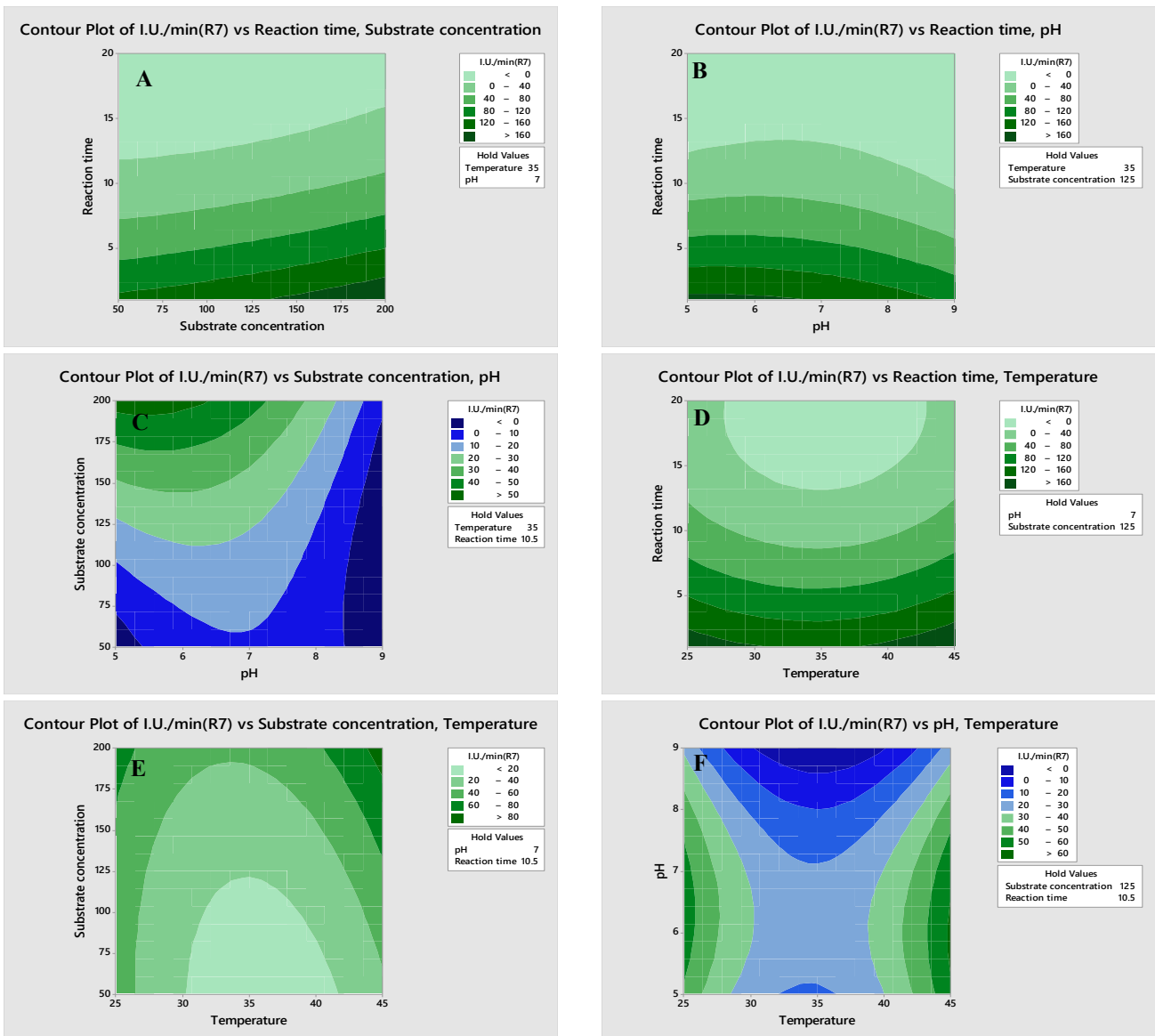

Fig. 4. Contour plots representing interactive effects of variable on the lipase activity by *Bacillus* sp. R7.

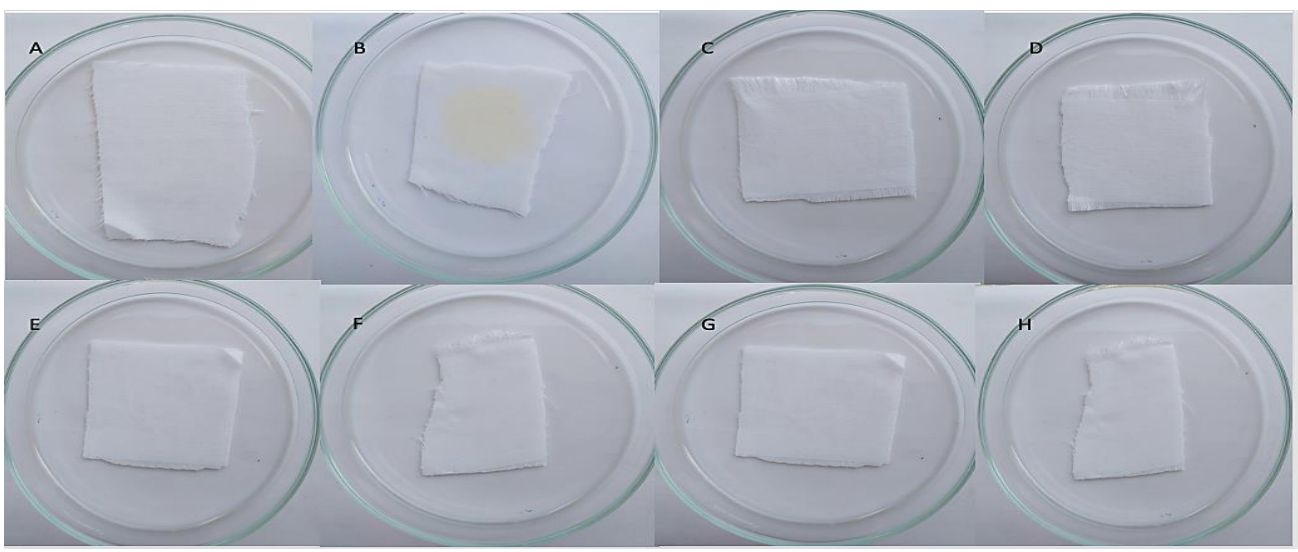

Fig. 5. Effect of lipase on stain removal. (a) Plain cloth (b) Olive oil soiled cotton (c) Washed with detergent after inactivating the enzyme (d) Washed without inactivating the enzyme (d) Washed with lipase from *Bacillus* sp. R7 prepared in buffer (f) Washed with lipase from *Bacillus* sp. R7 prepared in heat inactivated detergent (g) Washed with lipase from *B. licheniformis* E3 prepared in buffer (h) Washed with lipase from *B. licheniformis* E3 prepared in heat inactivated detergent.

The PBD design here, particularly investigated the type and/or level of nutrients in the medium that regulate the production of hydrolytic enzymes as these have an impact on the growth and metabolic rate of the producer (Nagar *et al.,* 2010). Davami *et al*., (2015) investigated the effect of various nitrogen sources, particularly different types of peptones on the growth of cell lines and found a positive correlation between the sources of optimal level with that of the higher growth. While Shariq & Sohail (2020) stated a positive impact of yeast extract on cellulase and xylanase production by *Candida tropicalis.* However, in this study, an appreciable difference of using organic or inorganic source over lipase production was not observed.

It has been reported that plant seeds may contain some inhibitory substances for bacterial growth and hence the nutrient composition needs to be determined specifically. Much earlier, (Satouchi *et al.,* 1974; Wang & Huang, 1984) it was reported that seeds of soybean contain some lipase inhibitors which were found to be protein in nature that attach to the surface of the substrate micelles and prevent lipase from performing normal function at the interfacial area. Later, it has been reported that the simultaneous supplementation of inducer with repressor such as simple nutritional sources inhibit the lipase production (Nagarajan *et al.,* 2014). Therefore, the impact of nitrogen and other nutrients need to be investigated carefully. It is also imperative to note that production of many hydrolytic enzymes is growth related and a higher volumetric productivity (Q_p) of cellulase production was positively correlated with the short generation time (Sohail *et al.,* 2009). Nonetheless, the growth of the bacteria is hampered in nutritionally rich medium with the association of higher rate of metabolites' production. Nonetheless, the investigations here resulted in 16-32 folds increase in the lipase production.

Lipase activity greatly varies depending upon the nature of oil-water interface. The properties vastly vary regarding fatty acid specificity, thermo-stability, pH optimum, type of substrate used and incubation time etc. Temperature can be considered as a source of activation energy and to provide the appropriate environment for the enzyme activity. Lipase enzymes have the temperature optima ranged from 30ºC to 60ºC (Sugihara *et al.,* 1991). Here, the lipase from the rhizospheric strain, R7, was most active at an elevated temperature (45ºC) than the lipase from the endophytic strain, E4, (25ºC) which can be linked with the natural habitat of the strains.

pH of the reaction mixture can alter the conformation of the enzyme which can lead to increase or decrease enzyme activity. Moreover, pH can also influence stability or solubility of the enzyme. The two lipases investigated here shared the optimal pH for their activity under acidic pH 5-5.3. The reaction time reflects the catalytic efficiency of the enzyme. In this study, a reaction time of one minute was found to be optimal. Previously, *B. licheniformis* H1 strain produced lipase with an optimal activity at pH 10 and 55ºC (Khyami-Horani, 1996). While a strain of *B. tequilensis* KP1-09 isolated from shrimp paste showed lipase activity ranged from 0.32 U mL^{-1} to 3.22 U mL^{-1} when the p-nitrophenyl palmitate (p-NP) was used as a substrate (Daroonpunt *et al.,* 2019).

The strategy used in this study by employing CCD was found promising and a considerable increase in the lipase titers from un-optimized conditions was noted. The lipase from *Bacillus* sp. R7 showed the highest activity, and its catalytic activity was increased from the initial activity of 96 IU mL^{-1} to 271.57 IU mL^{-1} showing almost 3-fold increase. *B. licheniformis* E3 lipase catalytic activity increased from 25.53 IU mL^{-1} to about 126.78 IU mL^{-1} showing ~5 folds increase from un-optimized assay conditions. The optimum condition studied here differed greatly from the lipase by *B. tequilensis* isolated from Thar desert that showed optimal activity at 60ºC at a pH ranged between 8-11 (Verma *et al.,* 2020). Nonetheless, the habitat of the microbes greatly influences the properties of their proteins.

Lipolytic enzymes must be suitable and stable with all commonly used detergent formulations in order to be effective under harsh laundry conditions (Zhao *et al.,* 2021). In the presence of commercial detergents, *B. licheniformis* E3 (32.42%) and *Bacillus* sp. R7 (43.74%) lipase demonstrated outstanding compatibility and stability (data not shown) that was in accordance with the lipase from *S. arlettae* (Chauhan *et al.,* 2013). Based on the current findings, the lipase preparation of *B. licheniformis* E3 and *Bacillus* sp. R7 in a buffer of pH 9 enhanced oil removal from soiled cotton fabric at 30°C with 100 rpm for 30 min that anticipated the application of lipase as a detergent additive. Lipases with thermostability and activity under alkaline conditions find various applications particularly in the detergent industry (Verma *et al.,* 2020).

Conclusion

The data presented enabled to conclude that the endophytic and rhizospheric bacteria associated with halophytes provide a prospect as resource for industrially important enzymes. The seeds of the halophytic plant, *A. macrostachyum* are source of lipids for production of lipase by *B. licheniformis* and *Bacillus* sp. A complete characterization of the seeds' constituent is needed in order to harness maximum benefits out of it. Statistical tools, Plackett-Burman design and Central Composite design are effective approaches to optimize the parameters affecting lipase production and characterization, respectively. The data showed that the lipases from the endophytic and rhizospheric strains are suitable to be applied as detergent additives.

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