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Screening and Isolation of an Organic Solvent Tolerant Lipase Producing Bacteria from Various Oil Contaminated Sites

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Abstract: Lipase producing microorganisms were isolated using tributyrin agar plate from different oil contaminated sites like petrol pumps, temple, kitchen waste dumping sites, etc. The plates were observed for zone of hydrolysis and C/Z ratio for each isolate was determined. The C/Z ratio of isolate C4 followed by U7 and K31 were the lowest and thus indicate larger clear zone as compared to colony. Potential lipase producing isolates were further screened for their solvent tolerance ability. Among them only seven isolates (C2, C3, C4, C7, K25, K31, and K35) were able to grow in the presence of organic solvents and also showed zone of hydrolysis on tributyrin agar in presence of organic solvents. The bacterial isolate K31was showed maximum growth in presence of almost all the organic solvents followed by K25 and K35 isolates. The aim of isolating a broad range organic solvent tolerant microorganism was fulfilled by isolate K31. The isolate K31 gave the highest activity with isopropanol (2.26±.15 U) at a solvent: media ratio of 1:3. The isolate K31 has also shown significant lipase activity with coconut oil (2.33±0.12 U) followed by olive oil (2±0.1 U), groundnut oil (1.76±0.14), soya oil (1.73±0.1).

Key words: Lipase, Tributyrin, Organic solvent tolerant microorganisms

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Introduction

Lipases (triacylglycerol acyl hydrolase; EC 3.1.1.3) are water-soluble enzymes that catalyze the hydrolysis of triacylglycerol to release free fatty acids and glycerol. They are serine hydrolase [1, 2]. However, lipase production from plants and animals leads to high cost and less yield [3] as a result microbial lipases have gained special industrial attention due to their stability, selectivity and broad substrate specificity [4]. A wide range of microorganisms (bacteria, fungi, yeasts) can produce lipases with different enzymatic properties and substrate specificities [5]. Its hydrolytic reaction is reversible in the presence of decreased amounts of water. Often in the presence of organic solvents, the enzymes are effective catalysts for various esterification, inter-esterification, acidolysis, alcoholysis and aminolysis [6, 7]. Most of the industrial reactions like esterification, transesterification, biodiesel production, and racemic resolution require lipases. For such kind of reactions lipase should retain activity in the presence of organic solvents. Furthermore, lipases that can function as catalysts in non-aqueous solvents offer new potentials like better solubility of substrate and product, shifting of thermodynamic equilibrium (synthesis takes place instead of hydrolysis), easy removal of solvent (most organic solvents have lower boiling point than water), reduction in water-dependent side reactions such as hydrolysis of acid anhydrides or polymerization of quinines, better thermal stability of enzymes since water is required to inactivate enzymes at high temperature, elimination of microbial contamination and potential of enzyme to be used directly within a chemical process [7]. However, organic solvents inactivate enzymes by decreasing conformational flexibility, stripping of crucial water, exposing hydrophobic residues, interfacial inactivation etc. Several strategies are available to overcome these limitations, which include chemical modification of amino acids on enzyme surface, protein engineering, medium engineering, use of ionic liquids (supercritical fluids) and co-lyophilization with non-buffer salts. Alternately, it has been proposed that instead of modifying enzyme for increasing solvent stability, it would be more desirable to screen for naturally evolved solvent-tolerant enzymes for application in non-aqueous enzymatic synthesis [8]. Hence, numerous organic solvent-tolerant lipases were isolated from different sources. Some of the well reported bacterial spp. are *P. aeruginosa* [9], *Bacillus sphaericus* [10], *Bacillus megaterium*[11], *Bacillus thermoleovorans* [12], *Burkholderia cepacia* [13], *Staphylococcus saprophyticus* [14] and some of the fungal spp. like *Penicillium chrysogenum* [15] and *Cryptococcus sp.*[16] are also effective producers.

Therefore, lipases are one of the most important industrial enzymes and applicable in many fields like food industry for flavour and aroma [17], pharma industry for kinetic resolution of compound [18], cosmetic industry, detergent industry for hydrolysis of oil, dairy industry for flavour [19] and fuel industry [20].

In the present study, isolation and screening of potential organic solvent tolerant lipase producing microorganisms and their characterization is discussed.

Materials and Methods

Chemicals and Media

Nutrient broth, agar powder, tributyrin, olive oil, groundnut oil, coconut oil, rice bran oil, soya oil, castor oil, cotton oil, corn oil, Glycerol, yeast extract, peptone, sodium chloride (NaCl), hydrochloric acid (HCl), magnesium sulphate ($MgSO_4$), Tris base, methanol, ethanol, isopropyl alcohol, acetone, chloroform, n-hexane, toluene, butanol, acetone, acetonitrile, ethyl acetate, benzene, xylene, cyclohexane, triton X 100 (Hi- Media, Mumbai), phenolphthalein indicator (Loba Chemi, Mumbai), p-NPP (para- nitophenol palmitate) were obtained from Sigma (St. Louis, MO, USA). Tributyrin agar media composition (g/l): Peptone (5), Yeast extract (3), Tributyrin (5ml), agar (30) and pH 7.5.

Methods

Isolation of lipase producing bacteria

Soil samples were collected from different sites like kitchen waste dumping site; petrol pumps oil rich sludge areas, etc. One gram of soil sample was suspended into 10 ml sterile distilled water and allowed to stand for some time to prepare a soil suspension. Suitable dilutions were prepared, transferred on tributyrin agar plates and incubated at 28^{o}C [15, 21]. Colonies with clear zone will be selected for further screening.

Primary screening of isolates for lipase production

The isolates obtained from different oil contaminated sites were further screened using tributyrin agar plate for lipase production. Upon 24-36 h incubation the plates were observed for the presence of clear zone due to hydrolysis of tributyrin which indicates the production of lipase enzyme. Colonies with large zone of hydrolysis and small C/Z ratio were selected for secondary screening (Figure 2 & Table 2).

Inoculum Development

The isolate was grown in the basal media containing component (g/l) yeast extract (20), $MgSO_4$ (2), NaCl (5) and olive oil (10 ml) which was incubated under shaking condition at 120 rpm at 37°C for 16 h. The attained microbial growth was used as inoculum for production of lipase after adjusting OD 1.0 at 600nm.

Secondary screening for solvent tolerant lipase production

Secondary screening was preceded by two methods, Plate-overlay method and Tube method.

i) Plate overlay method: This method was used to screen the solvent tolerant bacteria on tributyrin agar plate. Different organic solvents were chosen i.e. ethanol, chloroform, n-hexane, heptane, toluene, butanol, methanol, isopropanol, acetone, acetonitrile, ethyl acetate, benzene, xylene, and cyclohexane. Respective solvents were directly poured on the top of agar surface to

the depth of 5mm in each plate which was inoculated with selected bacterial isolates prior. All the plates were incubated at 28°C for 48 h and observed for clear zone. Volume of solvents was maintained throughout incubation period in the plates [30]. Bacterial cultures showing clear zone were further subjected to tube assay for solvent tolerance and lipase activity was determined [22].

ii) Tube method: Tubes containing 15 ml basal media broth were inoculated with different bacterial isolates which gave clear zone in presence of organic solvents on TBA plates. 2 ml of respective solvents were added into the tubes. All the tubes were incubated at 28°C for 48 h and microbial growth was determined spectrophotometrically at 600 nm. The isolates which gave significant growth in maximum number of solvents were selected as organic solvent tolerant lipase producing isolates and their biochemical characteristics were performed as per the procedure described in Bergey's Manual of Systematic Bacteriology.

Determination of lipase activity in presence of different organic solvents

Lipase production was carried out with selected isolates in Erlenmeyer flask with 50 ml of basal medium along with solvents. The flasks were inoculated with 1% inoculum and incubated at 28°C. Upon 24 h incubation samples were centrifuged at 8000 rpm for 5 min and the supernatant was taken for lipase assay.

Time profile for lipase production

Lipase production was carried out with selected isolates in Erlenmeyer flask with 50 ml of basal medium along with solvent. Flask was incubated at 37°C and at every 24 h lipase activity was determined as the method described by Cardirci and Yasa (2010) [22].

Optimization of solvent: media ratio for lipase production

Different solvent: media ratio was taken in varying range of 1:3, 1:2, 1:1, 1:0.2. Solvents with this ratio were mixed with basal media for lipase production. The flasks were inoculated with 1% inoculum and incubated at 37°C for 24 h at 100 rpm. The lipase activity was determined at 24 h time interval as the method described by Cardirci and Yasa (2010) [22].

Determination of lipase activity in presence of different oils

Medium was supplemented with 1% v/v concentration of different oils like rice bran oil, cotton oil, soya oil, corn oil, coconut oil, castor oil, ground nut oil, olive oil and glycerol [33]. The flasks were inoculated with 1% inoculum and incubated at 37°C at 100 rpm for 24 h. The lipase activity was determined at 24 h as the method described by Cardirci and Yasa (2010) [22].

Determination of lipase activity

Lipase activity was determined as per the method described by [22] using p-nitrophenyl palmitate (p-NPP) as a substrate. The substrate pNPP (30mg) dissolved in 10ml isopropanol and 90 ml Tris buffer (pH 7.2) in conjunction with TritonX-100 (0.4% (w/v)). The assay system consisted of 2.4 ml substrate and 0.1 ml of enzyme which incubated at 37^{o}C for 15 min. The absorbance was

measured at 410 nm. One unit (U) of lipase activity is defined as the amount of enzyme required to convert 1 μmol of pNPP to p-nitrophenol per min under specific conditions.

Results

Isolation and Screening of lipase producing bacteria

A total 19 bacterial isolates showing clear zone on tributyrin agar were isolated from different oil contaminated soil samples. Colony characteristics of all the isolates were studied (Table 1).

Table 1: Isolation and Screening of lipase producing microorganism

Sample code	Isolate Code	Size	Shape	Margin	Pigment	Opacity	Texture	Elevation
Kitchen waste soil-1	K12	Small	Round	Entire	Yellow	Opaque	Smooth	Convex
Kitchen waste soil-2	K25	Small	Round	Entire	White	Opaque	Rough	Flat
Kitchen waste soil-3	K31	Small	Round	Entire	White	Opaque	Smooth	Raise
	K32	Moderate	Irregular	Undulate	White	Opaque	Rough	Flat
	K35	Large	Irregular	Undulate	Cream	Opaque	Rough	Flat
	C1	Moderate	Round	Entire	White	Opaque	Smooth	Convex
	C2	Small	Round	Entire	White	Opaque	Rough	Flat
	C3	Small	Round	Entire	Yellow	Opaque	Smooth	Convex
	C4	Small	Round	Entire	White	Opaque	Smooth	Raise
	C5	Moderate	Round	Entire	White	Opaque	Smooth	Convex
	C6	Small	Round	Entire	Yellow	Opaque	Smooth	Convex
	C7	Small	Round	Entire	White	Opaque	Smooth	Flat
Temple	U1	Moderate	Round	Entire	Pale yellow	Opaque	Smooth	Convex
	U2	Small	Irregular	Undulate	White	Opaque	Rough	Convex
	U7	Small	Round	Entire	Orange	Opaque	Smooth	Convex

Garage soil	GA1	Small	Irregular	Undulate	White	Opaque	Smooth	Convex
	GA2	Moderate	Round	Entire	White	Opaque	Rough	Convex
Petrol pump	P7	Moderate	Round	Entire	Pale yellow	Opaque	Smooth	Convex
Kitchen waste soil-4	K41	Small	Irregular	Undulate	White	Opaque	Rough	Convex

Primary screening of isolates for lipase production

All 19 isolates were screened for their ability to produce lipase by spot inoculation on 1% tributyrin agar. Upon 24 h incubation at 28^{o}C, the plates were observed for clear zone surrounding the colonies and calculated C/Z ratio was calculated for each isolate (Table 2). The c/z ratio of isolate C4 followed by U7 and K31 were the lowest thus indicate larger clear zone as compared to colony.

Table 2: C/Z ratio of each isolate on tributyrin agar

Sr. No.	Isolates Code	Colony diameter (cm)	Zone diameter (cm)	C/Z
1	K12	0.8	1.2	0.66
2	K25	0.6	1.3	0.46
3	K31	0.3	0.7	0.42
4	K32	0.6	0.9	0.66
5	K35	1.0	1.3	0.76
6	C1	-	-	-
7	C2	0.5	0.8	0.62
8	C3	0.6	1.1	0.54
9	C4	0.4	1.2	0.33
10	C5	0.7	1.0	0.70
11	C6	0.6	0.9	0.66
12	C7	0.4	0.8	0.50
13	U1	0.4	0.7	0.57
14	U2	0.3	0.6	0.50
15	U7	0.5	1.3	0.38
16	GA1	0.7	1.3	0.53
17	GA2	1.2	1.5	0.80
18	P7	0.4	0.6	0.66
19	K41	0.5	0.9	0.55

Secondary screening for solvent tolerant lipase production

In case of plate assay, potential lipase producing isolates were screened for their solvent tolerance ability by spot inoculation on tributyrin agar. Among them only seven isolates (C2, C3, C4, C7, K25, K35, and K31) were able to grow in the presence of organic solvents and also showed clear zone on tributyrin agar. Thus, they were further confirmed by the tube assay.

In case of tube assay method, isolates (C2, C3, C4, C7, K25, K35, and K31) were further checked for their growth in presence of previously selected solvents. Isolate K31 was showed significant growth in presence of almost all the organic solvents followed by isolates K25 and K35. The growth was measured in terms of absorbance at 600 nm (Fig. 1A). The gram staining results of isolate K31, K25 and K35 indicate they are gram positive cocci and rods respectively and their zone of hydrolysis on tributyrin agar plates is shown in Fig. 1 (B). The bacterial isolates biochemical tests results are tabulated in Table 3. According to obtained results isolate K31 may be belong to genera *Staphylococcus*, isolate K25 may be belong to genera *Bacillus* and isolate K35 may be belong to genera *Lactobacillus*.

Fig. 1(A) Determination of growth of isolates in presence of different organic solvents

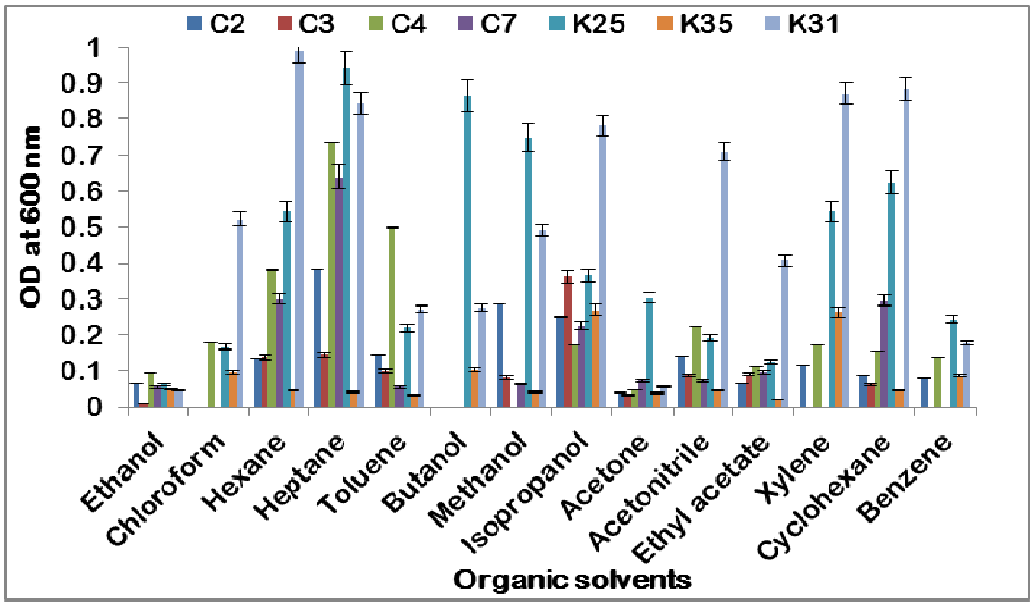


Fig. 1(B) Gram's staining and zone of hydrolysis of bacterial isolates a. K25, b. K35 and c. K31

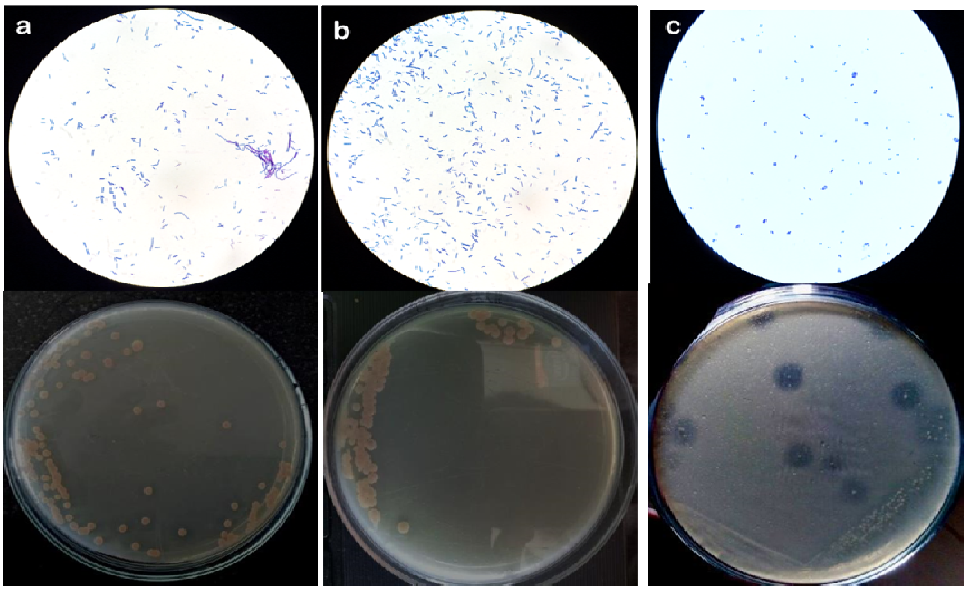


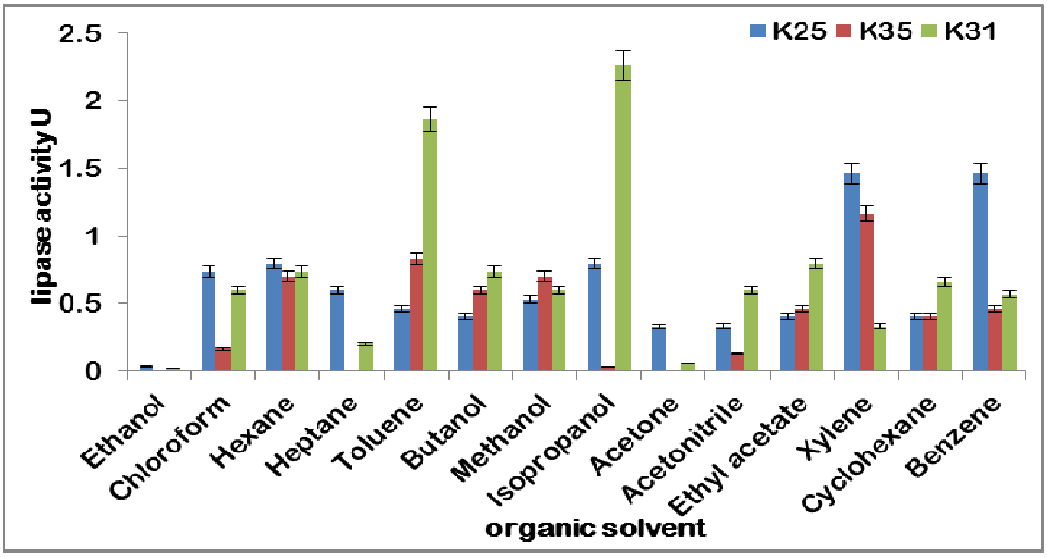
Table 3 Biochemical Characteristics of bacterial isolates K25, K35 and K31

Isolate	Gram's stain	Morphology	Spore stain	Capsule stain	Motility	Catalase Test	Citrate utilization	VP-Test	Casein hydrolysis	Starch hydrolysis	Glucose	Lactose	Maltose
K25	+ve	Rod	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
K35	+ve	Rod	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve
K31	+ve	cocci	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve

Determination of lipase activity in presence of different organic solvents

The isolates K31, K25 and K35 were showed growth in presence of most of the organic solvents. Hence, these isolates were allowed for lipase production in presence of different organic solvents (Fig. 2). Isolate K31 gave the highest activity (2.26±.15 U) with isopropanol followed by toluene (1.86±0.11 U) while isolate K35 gave maximum lipase activity (1.16±0.13U) with xylene. Whereas, the isolate K25 showed significant activity with benzene and xylene (1.46 U). The aim of isolating a broad range organic solvent tolerant microorganism was fulfilled by isolate K31.

Fig. 2 Determination of lipase activity in presence of different organic solvents



Time profile for lipase production

Lipase production profile was studied and at every 24 h time interval, broth was harvested to determine lipase activity. The highest activity (2.3±0.12U/ml) was observed at 24 h with further incubation lipase activity was declined at 48 h and 72 h (Fig. 3).

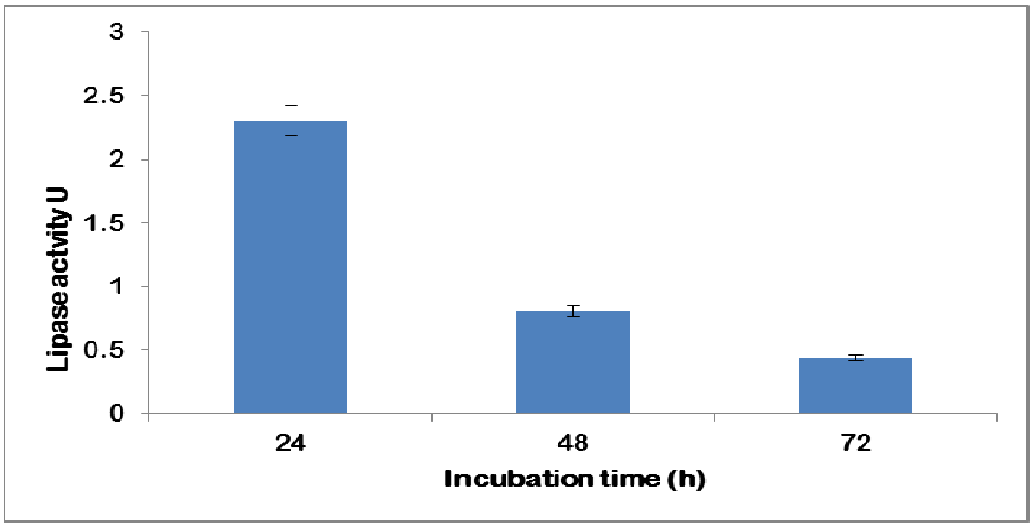
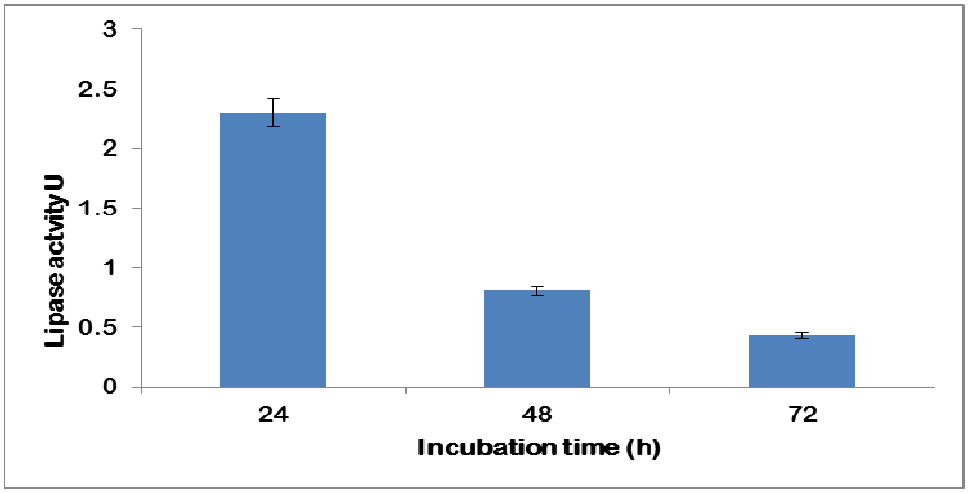


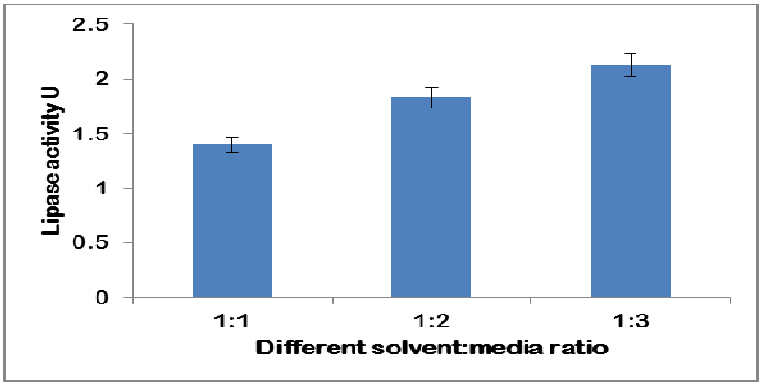
Fig. 3 Time profile for lipase production for bacterial isolate K31



Optimization of solvent: media ratio for lipase production

To optimize effective solvent media ratio for lipase production, different volume ratios of solvent and media were taken. As the isolate K31 showed highest stability and activity in isopropanol, variable ratio of isopropanol and media were analyzed. The isolate K31 gave the highest activity (2.1±0.16 U) with 1:3 ratio followed by 1:2 (1.83±0.12U), 1:1(1.4±0.1U) (Fig. 4).

Fig. 4 Effect of solvent: medium ratio on lipase production

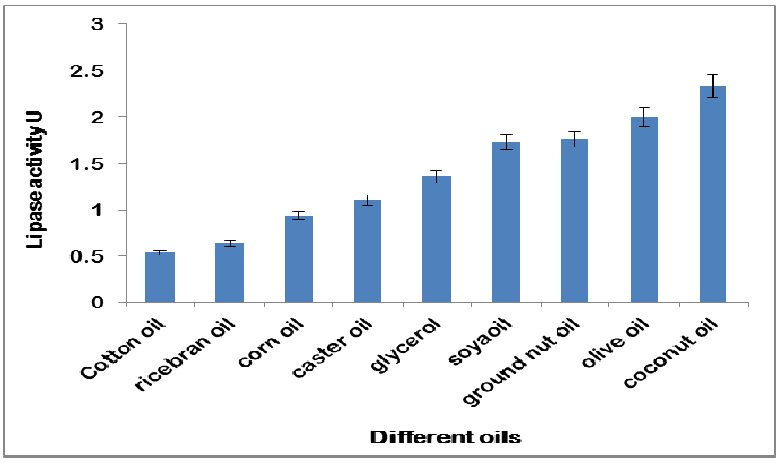


Determination of lipase activity in presence of different oils

To study the influence of oils on lipase production medium was supplemented with various oils as a carbon source. The bacterial isolate K31 gave the highest lipase activity with coconut oil

(2.33±0.12 U) followed by olive oil (2±0.1 U), groundnut oil (1.76±0.14), soya oil (1.73±0.1) and low activity was reported for glycerol, castor oil, corn oil, rice bran oil and cotton oil (Fig. 5).

Fig. 5 Determination of lipase activity in presence of different oils



Discussion:

Lipases are ubiquitous enzymes and produced by various plants, animals and microorganisms. Generally, lipases which are plant and animal origin have several limitations like extraction of enzymes is difficult, process cost is high, less stability, less yield, etc. [3]. Therefore, microbial lipases have gained special industrial attention due to their stability, selectivity and broad substrate specificity [23]. Extracellular lipase producing microorganisms can be mainly isolated from oil contaminated sites such as industrial wastes, vegetable oil processing factories, dairies, contaminated soil, oilseeds, decaying food, compost heaps, coal tips and hot springs [5]. The lipases have ability to preferentially hydrolyze long/short or saturated/unsaturated fatty acyl residues, but they also exhibit a positional specificity of a triacylglycerol molecule. Therefore, potential lipase producers are screened by direct observation methods in which zone of hydrolysis appeared around lipase producing colony due to hydrolysis of substrate like triolein [10], olive oil and tributyrin, etc [24]. However, in case of tween 80, opaque halos of calcium oleate appear in place of hydrolysis zones. In this study lipase producers were isolated from different oil contaminated sites and they were screened for lipase production using tributyrin agar medium. However, other than tributyrin, triolein [10], olive oil [25] or tween 20 agar [26] media can also be used for lipase producing microbes screening.

In addition, the ability of enzyme being active in the presence of organic solvents has received much attention due to apart from hydrolysis of triacylglycerol many reactions, like esterification, inter-esterification, acidolysis, alcoholysis and aminolysis [7] are reversible reaction of hydrolysis and occurred in decreased amounts of water and in presence of organic solvents [27]. In present study three isolates K31, K25 and K35 were screened as potential lipase producers in presence of

different organic solvents. Among them the isolate K31 showed highest lipase activity (2.26±0.15 U) with isopropanol followed by toluene (1.86±0.11 U). The isolate K35 gave maximum lipase activity (1.16±0.13U) with xylene whereas; the isolate K25 gave maximum activity with benzene and xylene (1.46 U). Similarly, Hun et al, 2003[30] reported 131 organic solvent tolerant isolates by direct plate method using 1% (v/v) of benzene, toluene or mixture of both. Out of them six isolates showed tolerance up to 75% (v/v) concentration of BTEX (benzene, toluene, ethyl benzene and xylene). Likewise, Torres *et al.,* 2011[14] reported *Staphylococcus haemolyticus* was able to grow in the presence of cyclohexane, benzene, and toluene in organic solvent plate overlays. Similarly, Dandavate *et al.,* 2009 [8] investigated solvent tolerance of bacteria by its ability to grow on tributyrin agar plates flooded with different solvents (n- hexane, isooctane, benzene, ethyl acetate, toluene, cyclohexane, dimethyl fluoride and dimethyl sulfoxide) during incubation. The culture exhibited growth as well as lipase production in presence of all solvents tested with maximum zone of hydrolysis on plates flooded with n-hexane and isooctane. Dheeman *et al.,* 2011 [28] reported that *Penicillium sp.* DS-39 (DSM 23773) was stable in the presence of non-polar hydrophobic solvents such as toluene, n-xylene, n-hexane, n-heptane, isooctane and dodecane. However, exposure to polar solvents methanol, ethanol, 2-propanol and n-butanol showed extreme reduction in growth as well as in lipase activity. Usually, lipase active site is covered by a lid like structure which makes the enzyme catalytically inactive. When lipase comes in contact with an interface between water and hydrophobic phase, the lid of the enzyme opens and active site of enzyme exposes for the binding of substrate [29]. In the presence of organic solvents enzyme interaction with non-polar substrates is enhanced and as a result overall yield of the enzyme is increased. Therefore, lipase is generally produced on lipid carbon sources such as oils, fatty acids, triglycerides, glycerol or tweens in supplementation of an organic nitrogen source. In reference to this Veerapagu *et al.,* 2013[5] reported different sugar media supplemented without oil source has given less lipase activity in comparison to media supplemented with olive oil. Similar line of observation was also illustrated by Kamini *et al.,* 2000[16], isolated extracellular lipase producer *Cryptococcus sp.* S-2 which gave significant yield of lipase in presence of certain lipidic carbon sources such as sardine oil, soy bean oil and triolein within 120 h at 25^{o}C. Hun *et al.,* 2003 [30] isolated organic solvent tolerant lipase producing *Bacillus sphaericus* and maximum lipase production (0.40 U) was observed within 24 h due to the presence of tween 80 and gum arabic in production medium. Hence, the search for organic solvent tolerant lipase has become an extensive area of research and it has been proposed that instead of modifying enzyme for increasing solvent stability, it would be more desirable to screen for naturally evolved solvent tolerant enzymes for application in non-aqueous enzymatic reactions [8].

Conclusion

Lipases are hydrolytic enzymes that hydrolyze triglycerides to fatty acids and glycerol at the oil–water interface, and catalyze the reverse reaction like esterification, transesterification and interesterification in non-aqueous solvent systems. Therefore, lipases have diverse applications in many biotechnological fields such as food, dairy, pharmaceutical and agrochemical industries. In

present study potential organic solvent tolerance lipase producers were isolated from different oil contaminated sites. Out of 19 isolates, the isolate K31, K25 and K35 were found as potential lipase producers in presence of different organic solvents. Among them isolate K31 gave maximum activity in presence of isopropanol with 1:3 ratio of solvent: media. Maximum lipase production was observed with coconut oil (2.33±0.12 U) followed by olive oil (2±0.1 U), groundnut oil (1.76±0.14), soya oil (1.73±0.1), glycerol, castor oil, olive oil, corn oil, rice bran oil and cotton oil. Hence, in the present study, the isolate K31 showed significant lipase activity between 0.5 to 2U in presence of isopropyl alcohol, n-hexane, toluene, butanol, ethyl acetate, benzene and cyclohexane. Therefore, bacterial isolate K31 lipase may be used for different esterification reactions.

Conflict of interest: The authors hereby want to declare that there is no conflict of interest whatsoever in this publication.

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