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Screening, isolation and production of lipase/esterase producing *Bacillus* sp. strain DVL2 and its potential evaluation in esterification and resolution reactions

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ABSTRACT

The isolate DVL2 was isolated from common city garbage using the tributyrin as substrate. The isolate resulted in orange fluoresce under UV light on rohodamine olive oil agar plate detecting the lipase production. Three production media (PM1, PM2 and PM3) were evaluated for lipase/esterase production. In culture filterate (extracellular enzyme) and cell free extract i.e. extract after sonication of cells (intracellular enzyme), both lipase and esterase activity were detected. But esterase activity was found to be associated only with bacterial cells. The maximum intracellular (112 IU/L) and extracellular (33 IU/L) lipase production were obtained in Production medium 2 after 24 and 36 h respectively whereas the maximum production of esterase (extracellular, intracellular and membrane bound) was obtained in Production medium 2 after 24 h. The DVL2 lipase/esterase was found to esterify stearic acid with ethanol resulting in the formation of ethyl stearate which was confirmed by thin layer chromatography. Furthermore DVL2 lipase gave positive results when applied for resolution of chiral auxillary viz. 1-acetyl phenyl ethanol.

Key-words: *Bacillus* sp., Lipase, Esterase, Esterification, Tributyrin Agar Plate Assay

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INTRODUCTION

Lipolytic enzymes [Esterase (E.C. 3.1.1.1) and Lipases (E.C. 3.1.1.3)] belong to a group of enzymes whose biological function is to catalyze the hydrolysis of triacylglycerols into diacylglycerols, monoacylglycerols, free fatty acids (FFA) and glycerol [1]. Many attempts were done by researchers to classify lipolytic and esterolytic enzymes [2]. Industrial applications may require specific enzymes like leather industry mostly require lipase and dairy industry require esterase [3]. Lipases and esterases can be distinguished on the basis of their substrate spectra, as esterases catalyze the hydrolysis of carboxylic ester bonds of short chain fatty acids (<10 carbon atoms) while true lipases have marked preference for long chain fatty acids (>10 carbon atoms) as substrates [4]. Lipase should be activated by the presence of an interface, that is, its activity should sharply increase as soon as the triglyceride substrate forms an emulsion.

Lipoytic enzymes are highly diversified in their industrial application. They have emerged as key enzymes which find usage in food, dairy, paper, textile, leather and detergent industries, waste water treatment, production of fine chemicals, pharmaceuticals and cosmetics, synthesis of surfactants and polymers, vegetable fermentation and meat product curing [5-10]. They can catalyze esterification, interesterification, and transesterification reactions in non-aqueous media with high chemo-, regio- and/or enantioselectivity [11,12]. Due to numerous biotechnological

applications, researchers have made attempts to isolate lipase/esterase producing microorganisms. Microbial lipases have received much more attention in industry mainly because of the availability of a wide range of hydrolytic and synthetic activities, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and easy cultivation of microbes on inexpensive media [13, 14]. Lipases of fungal and bacterial origin are widely used in industrial applications. Some important lipase producing bacterial genera include *Bacillus*, *Pseudomonas* and *Burkholderia* etc. Lipase/esterase-producing bacteria have been found in diverse habitats such as soil contaminated with oil, dairy waste, industrial wastes, oil seeds and decaying food, compost heaps, coal tips and hot springs [15,16]. The demand for the biocatalysts with novel and specific properties such as specificity, stability, pH, and temperature is increasing day by day [5, 17].

In view of the above facts, there is a great urge to explore novel lipases/esterases of industrial uses. The present study reports isolation and production of a lipase/esterase-producing bacterial strain DVL2 from soil samples and to evaluate its enzymatic potential in esterification of oleic acid for use in cosmetics and biodiesel production.

MATERIALS AND METHODS

Sample collection

Samples were taken from city common garbage site (comprising wastes of kitchens, restaurants, dairies, and sewerage and decaying plant parts) of the Karnal district of Haryana, India.

Isolation of lipase/esterase producing microorganisms

Samples were serially diluted with sterile distilled water and spread on the nutrient agar plates followed by incubation for 24-48 h at 37 °C for the growth of microorganisms. Microbial colonies, which appeared on nutrient agar plates, were purified and subjected to qualitative screening for identification of lipase/esterase producing micro-organisms on tributyrin agar (TBA), rhodamine olive oil (ROA), tween 20 and tween 80 agar plates.

TBA plate assay

Lipase/esterase producing micro-organisms produced a zone of clearance (hydrolysis) when their appropriate dilutions were spread on the TBA medium containing per liter of peptone, 5g; beef extract, 3g; tributyrin, 10ml and agar-agar, 20g. The zone size was measured after 24 and 48 h of incubation at 37°C.

ROA plate assay

A sensitive and specific plate assay for detection of lipase producing bacteria makes use of rhodamine-olive oil-agar medium [17]. The growth medium contained (g/L): nutrient broth, 8.0; NaCl, 4.0 and agar-agar 20. The medium was adjusted to pH 7.0, autoclaved and cooled to about 60 ºC. Then, 31.25 ml of olive oil and 10 ml of rhodamine B solution (1.0 mg/ml distilled water and sterilized by filtration) was added with vigorous stirring. It was then poured into petri plates under aseptic conditions and allowed to solidify. The bacterial culture was inoculated on to the medium in these plates. Lipase producing strains were identified on spread plates after incubation for 48 h at 37 °C. The hydrolysis of substrate causes the formation of orange fluorescent halos around bacterial colonies visible upon UV irradiation.

Tween 20/ tween 80 hydrolysis

The hydrolytic activity of *Bacillus sp.* DVL2 lipase was done on tween 20/ tween 80 medium composed of (g/L): peptone, 10; NaCl, 5; $CaCl_2.2H_2O$, 0.1; agar-agar, 20; tween 20 or tween 80, 10 mL (v/v).

Submerged fermentation

To study the effect of production media and incubation period, the isolate *Bacillus sp.* DVL2 was subjected to submerged fermentation in three different production media (PM1, PM2, PM3) for 24, 36 and 48 h. The composition of these production media is shown in Table 1. Inoculum size, pH of the production medium and incubation period selected for fermentation were optimum for the bacterial growth

Extraction of lipase

Lipase is extracted from the production medium after desired incubation time (24, 36 and 48 h) by centrifugation at 10000 x g for 30 min in a refrigerated centrifuge. The resulting supernatant contained extracellular lipase. The pellet was also collected and total cell biomass was calculated. The cell pellet was stored at –20^{o}C for further use. To release the intracellular lipase, 0.2g of harvested cells were suspended in 1 ml of lysis buffer (0.05 M phosphate buffer, pH 7) and subjected to five rounds of cell disruption (1 min) with the help of sonicator (MSE Manor Roya Crawley RH 10 2QQ) at 15 KHz for recovery of maximum enzyme. The sonicated cell suspension was centrifuged (15000 x *g* for 30 min) and cell free extract (intracellular lipase) was collected.

Table 1 Compositions of different production media (g/L)

Compositions of different production media (g/L)			
Components	**PM1**	**PM2**	**PM3**
Peptone	5	5	-
Beef extract	3	-	3
Yeast extract	-	3	-
Olive oil	1	-	1
Tributyrin	-	1	-
Tryptone	-	-	5
Glucose	3	-	-
Tween80	-	2	-
Sucrose			3

Enzyme Assay

Esterase/lipase assay (Titrimetric method)

The esterase activity was measured using modified titrimetric method as described by Beisson *et al*. [18]. The titrable solution containing 13.5 mL of 1% (v/v) tributyrin (substrate) in 1% gum acacia solution, 500µL of 2% calcium chloride and 1.0 mL of 1M NaCl. The reaction mixture was titrated against 10mM NaOH. The lipase activity was calculated using following formula: -

Lipase activity = Vol. of NaoH consumed (mL) × Molarity of NaoH/ Vol. of Lipase (mL) × Reaction Time (min).

One unit of lipase activity was defined as the amount of enzyme that liberated 1μmol fatty acid min^{-1} at 30 °C and pH 7 under the assay conditions.

Lipase assay

The activity of free lipase was determined spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as substrate according to the method of Nawani *et al.* [19] with some modifications. The reaction mixture containing 0.3 mL of 0.05M phosphate buffer (pH 8.0), 0.1 mL of 0.8 mM p-NPP and 0.1 mL of lipase was incubated at 37 °C for 10 min. The reaction was then terminated by adding 1 mL ethanol. A control was run simultaneously, which contained the same contents but the reaction was terminated prior to addition of the enzyme. Absorbance of the resulting yellow colored product was measured at 410 nm in a spectrophotometer. One International Unit (IU) of lipase activity was defined as the amount of enzyme catalyzing the release of 1 µmol of p-nitrophenol per min from p-NPP under the standard assay conditions.

Partial purification

The Extracellular lipase (culture filterate) was partially purified by ammonium sulphate fractionation for use in esterification. The culture filterate was subjected to protein fractionation by differential ammonium sulphate precipitation. Fractionation of protein was done by addition of small increments of solid ammonium sulphate at 4°C with constant stirring to obtain three fractions i.e 0-30%, 30- 60% and 60-90%. When all the ammonium sulphate was dissolved at the end of each fractionation range, the mixture was allowed to stand for 30 minutes to 1 h. The mixture was centrifuged at 10,000 x g for 30 minutes at 4º C. The pellet was collected and supernatant was used as starting material for next fractionation. The collected precipitate of each fractionation range was resuspended in small volume of 0.05M phosphate buffer pH 7.0 and checked for the enzyme activity as well as protein content.

Effect of the organic solvent

Organic solvent stability of DVL2 lipase was studied by incubating enzyme in different solvents (25% v/v) viz. isopropanol, xylene, methanol, ethanol, DMSO, toluene, hexane and acetonitrile, at 30°C for 12 h. The effect of different solvents was determined by determining residual activity of DVL2 lipase after 12 h.

Activity staining

Lipase/esterase activity was detected in polyacrylamide gel by activity staining Gabriel [20]. The enzyme sample was run on polyacrylamide gels (10%) under non-denaturing conditions in a cold room. After electrophoresis, gel was washed with 0.1M tris-HCl, pH 8.0 and then incubated it in staining solution [containing β-naphthyl acetate (8 mg/mL of absolute alcohol), fast blue RR salt (2 mg/mL) and 0.1M tris-HCl, pH 8.0 for 10-15 min at 37 °C till red coloured bands appeared. The β-naphthol released from the substrate β-naphthyl acetate couples with a diazonium salt present in the incubation mixture to form an insoluble red colored product visible as discrete bands.

Kinetic resolution of acetyl-1-phenyl ethanol

Kinetic resolution reaction was performed using a reaction mixture of substrate, 1-acetyl phenyl ethanol (25 mg/mL) in a 0.1 M phosphate buffer at 25 °C and partially purified lipase (0.75 IU) under stirred conditions. The

aliquots (100 µL) were withdrawn at different time intervals. The progress of reaction i.e resolution after different time interval (6, 12, 18, 24 and 30 h) was checked by TLC using ethyl acetate and hexane as solvent system.

Lipase/esterase catalyzed esterification

The partially purified lipase/esterase DVL 2 was used as biocatalyst for esterification of oleic acid with ethanol in 1:1 (v/v) ratio in hexane. The reaction was carried out at 37 °C with shaking at 100 rpm for 24 h with heat inactivated free enzyme (incubated at 100 °C for 5 min) as control. The ester formation was identified by analytical thin layer chromatography (TLC) performed using pre-coated silica gel 60 F_{254} MERCK (20x20 cm) plates. After chromatography, TLC plates were visualized by immersion in 10 % (v/v) H_2SO_4 in ethanol solution followed by heating on a hot plate.

RESULTS AND DISCUSSION

Screening and isolation of Bacillus sp. strain DVL 2

The sample collected from the city common garbage site was processed for isolation of a lipolytic bacterial strain *Bacillus* sp. DVL2 using qualitative and quantitative screening. The bacterial colonies on nutrient agar plates were subjected to qualitative screening on TBA for lipolytic strains [1,21]. Several colonies produced zone of hydrolysis on TBA. The zone of hydrolysis produced by the isolate DVL2 was shown in figure 1a. The zone size of this isolate on TBA increased from 2 to 5 mm as the time of incubation was increased from 24 to 48 h. The isolate DVL2 showing zone of hydrolysis on TBA can be regarded as esterase producer.

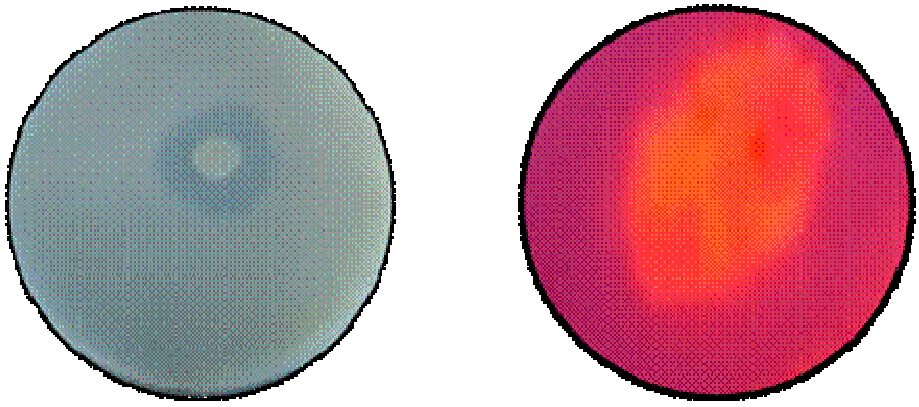

Figure 1 (a) TBA plate showing zone of hydrolysis (b) Rhodamine olive oil agar plate showing orange flouresence under UV light

To test DVL2 isolate as lipase producer, it was streaked on rhodamine-olive oil agar, tween 20 and tween 80 agar plates. The formation of orange fluorescent halos on rhodamine-olive oil agar plate under UV light by this strain (figure 1b) confirmed that it was also a lipase producer [21]. The florescence observed was due to reaction of the hydrolyzed substrate with rhodamine B. Fluorescent dye rhodamine B can also be used in plate assay containing emulsified olive oil to detect lipolytic organisms.

(a) **(b)**

Figure 2 Tween agar plates showing zone of precipitation produced by DVL 2 lipase/esterase (a) Tween 20 agar plate (b) Tween 80 agar plate

Tweens (fatty acid esters of polyoxyethylene sorbitan) have been the most widely used substrates for the detection of lipase/esterase producing microorganisms in agar media [22]. Screening using tween agar plates shows precipitation around the lipase/esterase producing micro-organisms. The method is based on the precipitation as the calcium salt of the fatty acids released by hydrolysis of tweens. Liberated fatty acids bind with the calcium incorporated into the medium. The calcium complex is visible as insoluble crystals around the inoculation site. Tween 80 is mostly hydrolyzed by the lipase as it contains esters of oleic acid and rarely by esterases while tween 20 is easily hydrolyzed by esterase as it contains esters of lower chain fatty acids viz. lauric acid. In the present study, formation of zone of precipitation on Tween 20 and Tween 80 agar plates by the isolate DVL2 (figure 2a, b) also confirmed that this bacterial strain produced both esterase and lipase.

Many researchers have isolated the lipase/esterase producing microbes from the different sources. A cold-adapted lipase producing strain was isolated from decayed seeds of *Ginkgo biloba by* Yuan *et al.* [23] Highly thermostable extracellular lipase producing *Bacillus* strain isolated from a Malaysian hot spring and identified using 16S rRNA gene sequencing by Akanbi *et al* [24].

Production media and incubation time

The isolate DVL2 was subjected to submerged fermentation in three different production media (PM1, PM2 and PM3) for varying time intervals followed by determination of esterase and lipase activities as well as biomass production (figure 3). Bacterial isolate DVL2 displayed lipase activity (extracellular and intracellular) as well as esterase activity (extracellular, intracellular and membrane bound) thereby supporting the results of qualitative analysis. Isolate DVL2 produced all three types of esterase activity i.e. extracellular (9.2 U/L), intracellular (5200 U/L) and cell bound esterase (60 U/g) maximally in PM2 after 24 h of incubation. The maximum extracellular lipase activity 33 IU/L was obtained after 36 h in PM2 whereas maximum intracellular lipase activity viz.112 IU/L after 24 h in the same production medium. Ertugrul *et al.* [3] isolated 17 bacterial strains from oil mill waste. Among these, a strain of *Bacillus* sp. was identified as the best lipase producer and after the medium optimization, maximum intracellular activity obtained was 168 U/mL.

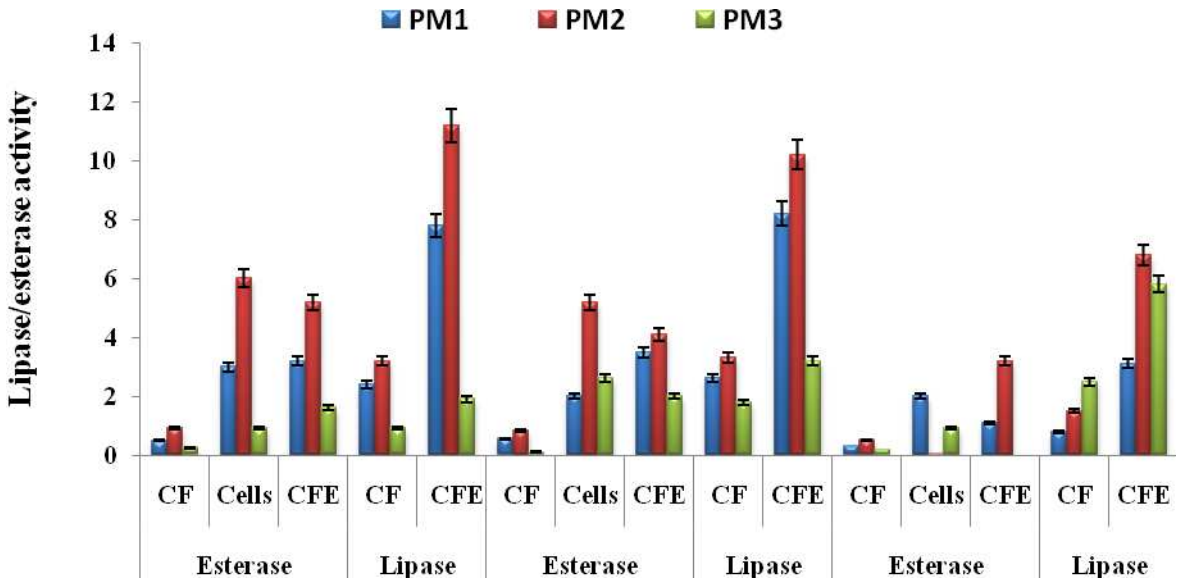

Figure 3 Quantitative measurement of lipase/esterase production on three different Production media (PM1, PM2 and PM3). CF and CFC represent culture filtrate (extracellular) and cell free extract (Intracellular lipase) respectively. The activity of esterase is expressed as: CF (IU/100ml); Cell pellet (IU/100mg) and CFC (IU/mL) whereas activity of lipase is expressed as: CF (IU/100mL); CFC (IU/100mL).

Effect of organic solvents

The stability of *Bacillus* sp. strain DVL2 lipase was determined after pre-incubation in various organic solvents for 12 h. DVL 2 lipase was found to be 100% stable in xylene, ethanol, DMSO and toluene (figure 4a). However DMSO and toluene appeared to have some activating effect on lipase activity. The DVL 2 lipase was found to be stable in both polar as well as nopolar solvents. The stability in water miscible solvents like ethanol and methanol, might be due to the maintaince of active conformation of enzyme. Similar to DVL 2 lipase, lipase from *Bacillus* sp. strain 42 exhibited high levels of activity in the presence of p-xylene, benzene, toluene [25].

Partial purification using ammonium sulphate fractionation

Most of the lipase activity was recovered at 30-60% saturation. This partially purified lipase had specific activity of 0.39 IU mg^{-1} protein with the total activity of 10.5 IU. The recovery of the enzyme was approximately 62.5 % with the purification fold of 18.5 as shown in Table 2.

Table 2: Partial purification table of the Extracellular lipase from *Bacillus sp.* DVL2

Fraction	Vol (mL)	Activity (IU/ml)	Total activity (IU)	Protein (mg/ml)	Total protein (mg)	Specific activity (IU/mg)	Purification fold
Crude	200	0.084	16.8	4	800	0.021	1
A.S. fraction [a] (30-60%)	7	1.5	10.5	3.77	26.4	0.39	18.5

[a] **Ammonium sulphate fractionation**

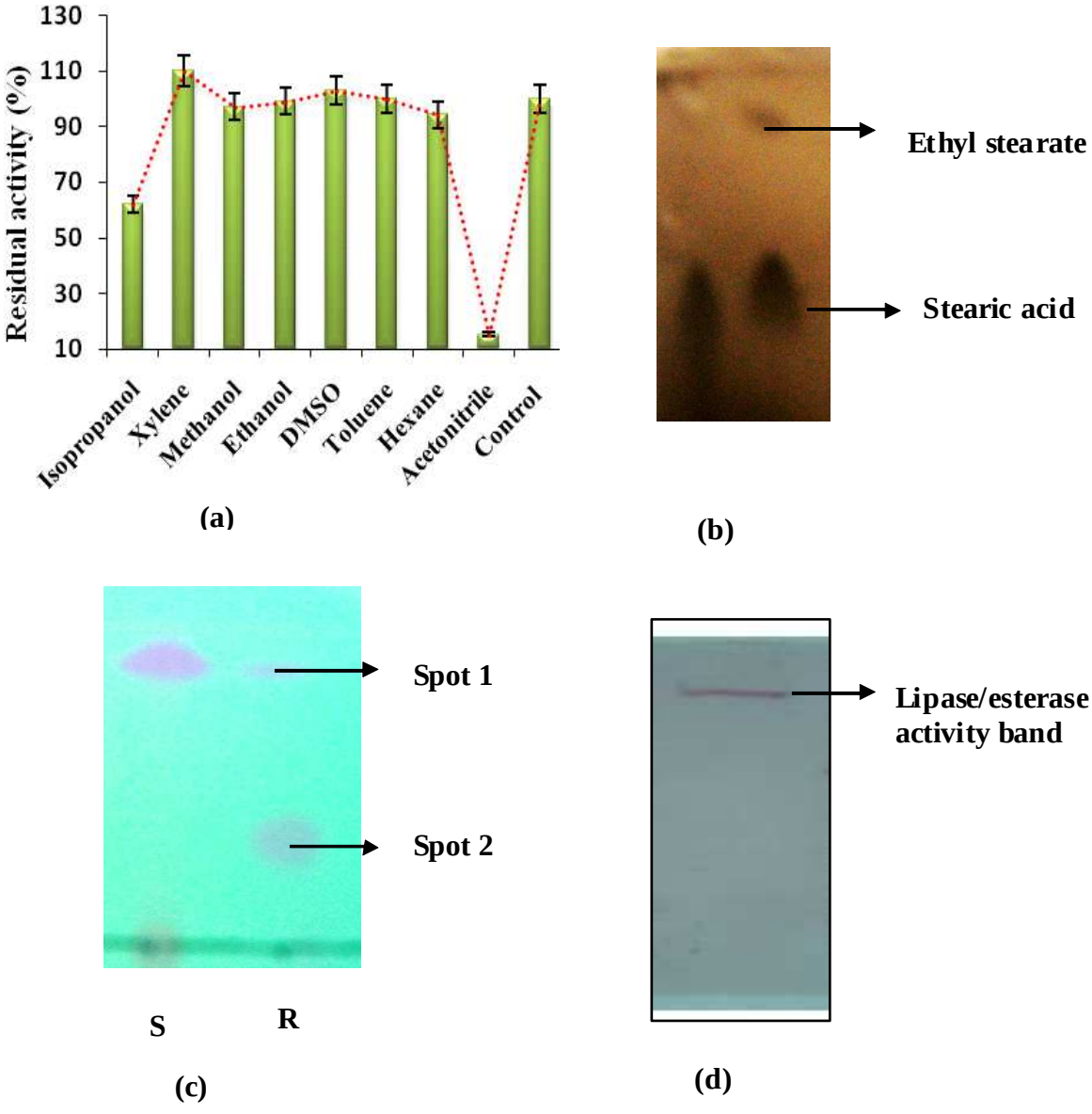

Figure 4 (a) Effect of various organic solvents (25% v/v) on DVL2 lipase (b) TLC plate showing esterification of stearic acid (c) TLC showing resolution of acetyl-1-phenyl ethanol under UV light (S: substrate; R: Reaction) (d) Activity staining showing lipase/esterase activity using Native –Page electrophoresis

Esterification

The isolate *Bacillus sp.* DVL2 was found to esterify stearic acid, with ethanol resulting in the formation of the ethyl stearate which was confirmed by thin layer chromatography (figure 4b). Esters of stearic acid are useful for cosmetic industry. Enzymatic synthesis of esters is always preferable over chemical synthesis as it is single step and free from the contamination of other chemicals which make the purification of ester multistep and expensive.

Resolution of acetyl 1-phenyl ethanol

The progress of resolution reaction by DVL2 lipase, with time (6, 12, 18, 24 and 28h) was monitored by TLC analysis. The 18 h old fraction showed the better resolution as indicated by two spots in the reaction mixture TLC in comparison to standard (acetyl 1-phenyl ethanol) TLC which had only one spot under UV ligh (figure 4c). This is just the preliminary evaluation which indicated that DVL2 lipase might be useful in the resolution work of chiral auxillary acetyl 1-phenyl ethanol and thus further research work could be planned.

Zymogram

Figure 4c represents activity staining for the DVL 2 lipase/esterase in which insoluble red colored product was visible as discrete bands in native page when treated with β-naphthyl acetate and fast blue RR salt. The β-naphthol released from the substrate β-naphthyl acetate couples with a diazonium salt present in the incubation mixture to form an insoluble red colored product visible as discrete bands [20]. A number of researchers have described methods for activity staining of lipases where either fluorescent substrates, substrate analogs, or various esters have been employed [26]. However, most of these substrates are expensive. Among these the most preferred substrates remain to be naphthyl acetates [27].

CONCLUSION

The bacterial strain *Bacillus* sp. strain DVL2 has been shown to exhibit both lipase and esterase activities on the basis of the results of qualitative and quantitative analysis. Lipase and esterase enzymes are in great demand these days owing to their numerous biotechnological applications**.** The lipase produced by DVL2 was found to esterify of stearic acid with alcohol. Esters of stearic acids are useful for cosmetic industry. High chemo-, regio- and stereo-selectivity have made lipase/esterase most important enzyme for pharmaceutical application like resolution of drugs.

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