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Isolation, characterization and antioxidant activity of lipase enzyme producing yeasts isolated from spoiled sweet sample

Aswathy Raj, Gabylis Baby, Sandip Dutta, Anwesha Sarkar and K. V. Bhaskara Rao*

Molecular and Microbiology Laboratory, Department of Biomedical Sciences, School of Biosciences and Technology, VIT University, Vellore, Tamil nadu-632014, India

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ABSTRACT

Yeasts are eukaryotic unicellular organisms and constitute 1% of all described fungal species. Many yeast species are capable of producing different types of bioactive compounds including enzymes viz. amylases, proteases, invertases, lipases, inulinases, cellulose, phytases. The present study was focused on the isolation of lipase producing yeasts from spoiled milk sweet sample collected from Vellore, Tamil Nadu sweet shops. A total of 18 different isolates were recovered from that sample, out of which 14 isolates were selected for primary screening based on distinct colony morphology. Primary screening of the selected yeast isolates was done in tributyrin agar plates where maximum clear zones of inhibition was about 14mm and 18mm respectively, indicating the production of the enzyme lipase. Higher enzyme activity showing 2 isolates were selected and sub-cultured for further. Those 2 yeast isolates were designated as SAGB1 and SAGB2.Secondary screening was done through assay with Phosphate and Tris-HCl buffers. SAGB1 and SAGB2 showed activity 60% and 65% respectively. However, in Tris-HCl buffer they showed activity 58% and 62%. SAGB1 and SAGB2 showed DPPH antioxidant activity of 53.9% and 48.93% respectively. The enzyme was extracted using different solvents as means of partial purification.

Key words: Marine yeast, Lipase, Isolation, Screening, Biochemical characterization, Antioxidant activity.

INTRODUCTION

The use and discovery of enzyme-mediated processes have been started since ancient civilizations. In today's world, approximately 4000 enzymes have been discovered, out of these, about 200 enzymes are of commercial use [1]. Lipases are also called as triacylglycerol acylhydrolases, (EC 3.1.1.3). They are actually carboxylesterases and they catalyse the hydrolysis of acylglycerides with acyl chains. They are type of serine hydrolases. Many of these enzymes are active in organic solvents for catalysing a number of important and useful reactions including esterification [2, 3]. While Lipases catalyse the hydrolysis of fats into fatty acids and glycerol at the oil water interphase, interestingly in non-aqueous media, they reverse this reaction [4, 5]. Lipases are activated when adsorbed to an oil-water interface and they do not hydrolyse dissolved in the bulk fluid [6]. It is already known that the sources of lipases are widely found in nature such as industrial wastes, decayed and spoiled food, dairies, oilseeds and oil contaminated soil [7] however, the lipases obtained from microorganisms only have the commercial values [8]. Commercially important lipases are generally produced from microorganisms specifically various species of bacteria, yeast and fungi. Microbial lipases are produced by both the submerged culture and solid-state fermentation methods [9, 10]. Several yeasts have been proven to be producer of lipase enzymes. Most used commercial producers of these enzymes are *Candida sp.; Yarrowia lypolytica* etc. [11] but *Saccharomyces sp.* and *Pichia sp.* have also been proved to produce lipases [12].

There are various functions of lipase enzyme. The research of lipases is mainly focused on structural characterization, kinetics, elucidation of mechanism of action, sequencing and cloning of lipase genes and some general functions and characterization [13]. It is applied clinically as an indicator determining the presence of lipids

in biosensors which can be chemical or electronic in nature [14]. Aroma of products can be improved by the use of lipases in beverages and food industries. Also in cosmetics production field, this enzyme is used as an emulsifier and moisturizer [15]. In addition to ester formation, lipases are developed as catalyst in chemical industry and the esters contribute in flavouring agents in food industry [16]. Lipases are used to produce ϒ-linolenic acid, a polyunsaturated fatty acid (PUFA); dicarboxylic acids as prepolymers; methyl ketones and astaxanthine, a food colorant [17]. Lipases are useful in the production of biodiesel and in the synthesis of biopolymers, enantiopure pharmaceuticals and agrochemicals. Due to its excellent chemo selectivity, regioselectivity and stereoselectivity, lipase enzyme is getting a decent amount of attention among researchers [8]. Lipases also play a major role in the field of bioremediation. Bioremediation is a microorganism mediated transformation or degradation of contaminants in to non-hazardous or less-hazardous substances [18]. Only certain species of fungi and bacteria have proven their ability as potent pollutant degraders. There is a close relationship with lipases and organic pollutants present in the soil. Lipase activity was responsible for the drastic reduction total hydrocarbon from contaminated soil. Oil spill pollution results in the release of liquid petroleum hydrocarbon into the environment [19].

Yeasts are single celled eukaryotic organisms and are a type of fungi which are larger than bacteria and are regarded as the 'model organism' [20]. The term yeast is derived from the German word 'gischt' and the Dutch word 'gist' meaning fermentation [21]. They are basically polyphyletic group of basidiomycetes and ascomycetes fungi and they also have a unique characteristic of unicellular growth [22]. Till now, approximately 100 genera and 800 species of yeast have been described. These numbers represent only 1% of the total yeast species and the rest is unknown [23]. Another important characteristic of yeast essential to their role as 'model organisms' is the fact they are relatively easy to work with. Yeast occurs in different forms including spherical, cylindrical, oval, pear-shaped etc. The major sources of yeast include plants, fruits, grains and sugar containing foods [24]. Terrestrial yeasts can be also found in various places including air, soil, skin and in the intestines of animals and certain insects. Yeasts are beneficial to food industry although some cause diseases and food spoilage [25].

It has been reported that yeast can produce various bioactive substances like glucans, killer toxins, glutathione, vitamins etc. [26]. There are reports of production of medicinal protoberberine alkaloids, bioactive molecules used in traditional medicines by optimizing a biosynthetic pathway in *Saccharomyces cerevisiae* [27]. They also have some potential applications in food, pharmaceutical, chemical and cosmetic industries as well as environmental protection [28]. Another important capability of yeast is enzyme production. Besides lipases, the most common enzymes produced by yeast are amylase, invertase, acid protease, alkaline protease, phytase, inulinase, and cellulase. Food spoilage basically means the original nutritional value, texture and flavour of the food are damaged and the food has become harmful and unsuitable for humans consumption [29]. Yeasts and moulds are of main problem in the case of spoilage in bakery and confectionary products [30]. Among all of the yeast species, *Saccharomyces cerevisiae*, *Saccharomyces dairensis*, *Saccharomyces exiguous* and *Pichia membranaefaciens* play a major role in the food spoilage [31].

Anti-oxidants can scavenge and inhibit free radicals which generally are able to oxidize nucleic acids, lipids, proteins and so they can initiate degenerative diseases. Most of the antioxidant compounds in a proper diet are mainly derived from plant sources and they belong to various classes of compounds with a wide variety of physical and chemical properties [32]. Antioxidants have a number of applications as dietary supplements, prevention of diseases like cancer, coronary heart disease etc. They also can be used in industrial purposes like as food preservatives, in cosmetics industry etc. [33].

The purpose of the present study is to isolate lipolytic yeasts, their antioxidant activity and also partial purification of the enzyme.

MATERIALS AND METHODS

Chemicals:
Yeast-malt agar medium, tributyrin agar, tributyrin oil, phenyl acetate, phenol, tris-Hcl, triton X-100, folin ciocalteu reagent purchased from Hi Media Laboratories Pvt. Ltd and Sisco Research laboratories Pvt. Ltd. (SRL).

Sample collection:
The spoiled milk-sweet sample was collected from a sweet shop in Vellore, Tamil Nadu. The sample was then taken to Molecular and Microbiology Laboratory, VIT University, Vellore and stored in the refrigerator at 4°C for further use.

Isolation of yeast:
Isolation of yeast was done by serial dilution and spread plate technique. A 10-fold serial dilution series was made with the sample which was followed by plating those dilutions in petriplates. Yeast-malt agar (YMA) medium was used for isolation of yeasts [26]. The media were finally supplemented with 100 mg/l chloramphenicol and 0.015 mg/l nalidixic acid to avoid bacterial and fungal growth. From each dilution, 0.1 ml of suspension was aseptically transferred to the corresponding petri plates. Then spreading was done thoroughly using glass spreader and plates were incubated at room temperature for 3-5 days.

Screening for lipase enzyme producing yeast isolates:
Pure cultures from the master plates were selected based on colony morphology and streaked on to fresh YMA plates under aseptic conditions. The plates were kept in the incubated for growth of yeast at room temperature for 3-5 days. The pure cultures obtained were screened for the production of lipase enzyme. Lipase activity in those yeast isolates was identified by the formation of clear zones around the yeast culture on tributyrin agar plates.

a) Primary screening for lipase (TBA method):
Primary screening was done using tributyrin agar plate method (TBA). A total of 14 yeasts isolates were selected based on the difference in colony morphology and they were streaked onto TBA plates. The plates were incubated at room temperature for 48 h. Yeast isolates capable of producing lipase will form transparent halo zone around the colonies which will prove positive result [34].

b) Secondary screening for lipase (assay):
Isolates showing positive results in primary screening were taken for further secondary screening. Production media containing (g/l) 0.3 yeast extract, 0.3 malt extract, 0.5 peptone and 1 glucose were prepared and the isolates were inoculated in that media and kept at room temperature in shocking condition for 3-5 days. After incubation period is over, the cultures were centrifuged at 8000rpm for 15 minutes and supernatant of each culture was taken ascetically.

Lipase Assay:
A substrate solution for the lipase assay was prepared with a buffer solution containing 1% triton X-100, a weight of phenyl acetate was dissolved and the final concentration was made 200µM. For the assay, 113.3 mg of phenyl acetate was dissolved in 10 ml of Tris-HCl buffer with pH 7 for preparing different substrate tubes of varying concentration from 0-200 µM. Then 0, 0.5, 1, 1.5, 2 and 2.5 ml were taken from the solution and equalised all tubes by adding 2.5 ml distilled water. 0.1 ml of lipase enzyme was added and activity was measured calorimetrically [35].

Identification and characterization of potential yeast strain:
a. Colony morphology:
The colony morphology was noted with respect to colour, shape, consistency, elevation and opacity of the colonies.

b. Microscopic observation:
Microscopic methods like gram staining and chlamydospore staining for spore identification were done to determine the isolates as yeast. For chlamydospore staining, the tentative yeast colonies were streaked on deeply cut corn meal agar plates supplemented with 1% Tween 80, concealed with a cover slip and incubated at room temperature for 3-4 days [36].

c. Biochemical characterization:
Biochemical tests like indole, methyl red, voges proskauer, citrate utilization test, oxidase, catalase tests and carbohydrate fermentation tests were done for characterization of the isolates.

Extraction of Lipase:
The enzyme was extracted using different solvents. Methanol, ethyl acetate, petroleum ether and chloroform (from polar to non-polar) were used and mixed with the culture filtrates (1:1 v/v). The solvent mixture was shaken vigorously for some time and after 24 h the phase which contained the extracted compound was separated from the other phase [37]. The extracts were dried using a rotary evaporator.

Antioxidant assay:
With the crude enzyme and partially purified extracts, antioxidant assay was performed to check the enzyme activity.

DPPH radical scavenging activity:
Yeast cells were centrifuged at 8000rpm for 15 minutes and supernatants were collected for DPPH assay. In test tubes, 1ml of supernatant from each strain was taken and then 2ml DPPH reagent (2, 2-diphennyl-1-picrylhydrazyl) was added. Test tubes were incubated in dark condition for 20 minutes at room temperature. The degree of reduction of the DPPH reagent was measured in UV-vis spectrophotometer as absorbance at 517 nm [38].

% Scavenging rate= (Control-Absorbance of test/Control) ×100

RESULTS AND DISCUSSION

Isolation of yeast:
A total of 18 yeast isolates could be isolated from the spoiled sweet sample. From those, 14 isolates were selected for primary screening based on their colony morphology. Those were sub-cultured and pure cultures were maintained properly in the same YMA media.

Primary screening of yeast isolates:
A total of 14 isolates were screened to check the lipase activity. Clear zones were observed around two of them indicating a positive result for screening [39]. The two yeast isolates were named as SAGB1 and SAGB2. SAGB1 and SAGB2 produced zones of clearance of about 14mm and 18mm respectively [Fig 1: (a) and (b)].

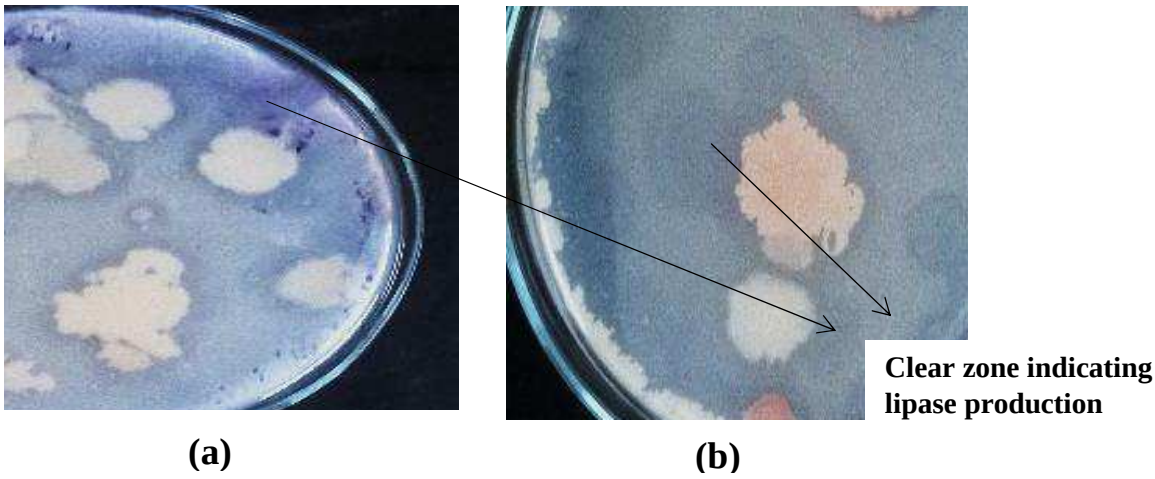

Fig.1: a) SAGB1 and b) SAGB2: formation of clear zones around skim milk agar plates

Secondary screening of yeast isolates:
Secondary screening was done calorimetrically. The lipase activity was determined for both the yeast isolates (SAGB1 and SAGB2). SAGB1 showed 60% activity and 65% activity was shown by SAGB2 in phosphate buffer. Besides, SAGB1 and SAGB2 showed 58% and 62% activity, respectively in Tris-Hcl buffer (Fig: 2 and Fig: 3).

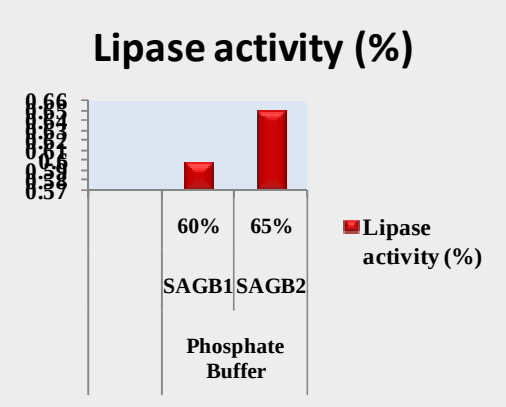

Fig 2: Activity of enzyme in phosphate buffer

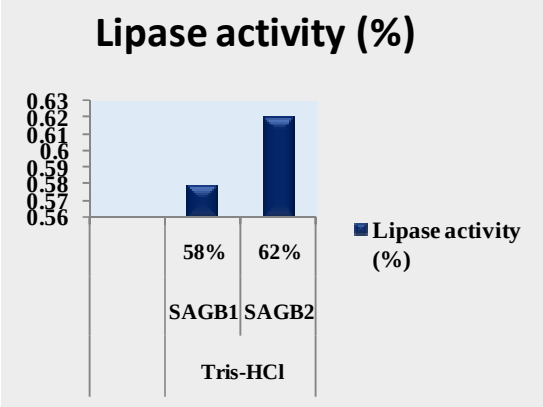

Fig 3: Activity of lipase enzyme in Tris-HCl buffer

In a previous study by Mohamed et al., [35] showed that in phosphate buffer activity was 0.09 U/ml and in Tris-HCl it was 0.16 U/ml. Those results are similar to the present study.

Characterization of potential isolates:

a. Colony morphology:

In macroscopic observation the two yeast isolates were round, circular in shape. SAG1 and SAG2 were white and creamy coloured respectively. Both the isolates were convex, slimy and opaque (table. 1).

Table 1. Colony morphology of yeast isolates

Isolates	Shape	Colour	Elevation	Consistency	Opacity
SAGB1	Round	White	Convex	Slimy	Opaque
SAGB2	Round	Creamy	Raised	Slimy	Opaque

b. Microscopic observation:

Gram staining technique revealed both the isolates were yeast with large budding cells, which were coloured purple (Fig 4) and showed positive results. Chlamydospore formation was not seen so we can tell that these isolates are not *Candida sp.* because chlamydospore staining is mainly for Candida sp. [36].

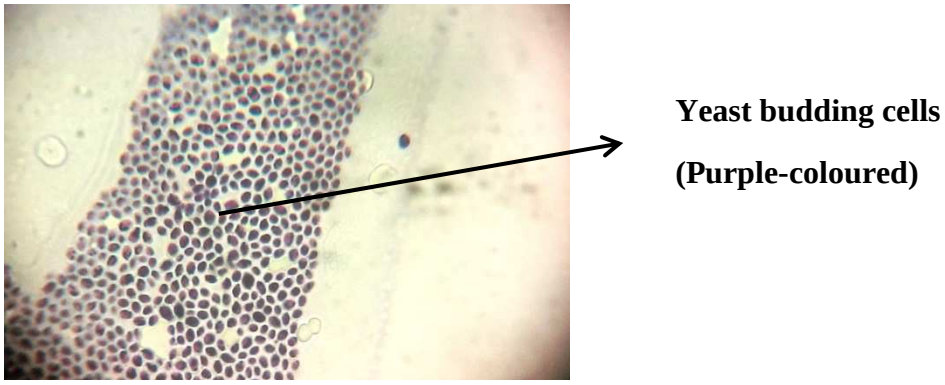

Fig 4: Gram-staining of yeast isolate SAGB2

c. Biochemical Tests:

The biochemical tests, including indole, methyl red, voges-proskauer, citrate utilization, catalase and oxidase test were done. SAG1 and SAG2 showed negative results for VP and citrate utilization test. The two yeast strains were positive for the rest of the tests. Both of the strains were positive for glucose fermentation also (Table.2). Carbon assimilation and carbon fermentation tests are there to determine the ability to use substrates as the sole carbon source [40]. Normally, *Saccharomyces sp.* can ferment glucose and some other carbohydrate vigorously [41]. Hence, by the microscopic observation and the results of biochemical tests we can assume that those potent yeast isolates can be *Saccharomyces sp.*

Table 2. Biochemical characteristics of yeast isolates

Tests	SAGB1	SAGB2
Indole	+	+
MR	+	+
VP	-	-
Citrate	-	-
Oxidase	+	+
Catalase	+	+
Glucose fermentation	+	+
Galactose Fermentation	+	+

Antioxidant activity:

DPPH assay:

The magnitude of anti-oxidation ability of examined sample can be expressed by their ability to scavenge DPPH radical. The DPPH reagent is actually violet in colour and biologically free radical. The change of colour from violet to yellow, hence indicates a positive test [42]. From the obtained result, we can see that SAGB1 showed 53.9% of activity, whereas SAGB2 showed 48.93% of DPPH activity (Fig: 5). In [43], they have done the total antioxidant activity of *Saccharomyces cerevisiae* and found maximum activity in the value 0.81 mmol TE/g. From this study we can also get to know that *Saccharomyces sp*. has antioxidant activity so our isolates might be belonged to this genus.

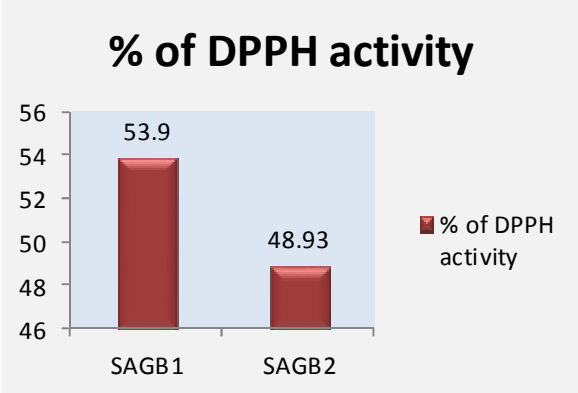

Fig 5: DPPH scavenging activity of yeast isolates

CONCLUSION

In the above study, lipase producing yeasts were isolated from spoiled milk-sweet sample. Among 14 isolates, 2 yeast isolates showed good lipase enzyme activity. After the partial purification through solvent extraction, antioxidant activity was also done and both the isolates showed good activity. Thus, these two yeast isolates SAGB1 and SAGB2 were proved to be very potential for lipase production. Among these 2 potential isolates, SAGB2 showed better enzyme activity than SAGB1. On the contrary, SAGB1 showed better DPPH scavenging activity than SAGB2. Future prospects include complete purification of the lipase enzyme and molecular identification of the isolates.

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