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Isolation of Novel Lipase Producing *Sporobolomyces salmonicolor* OVS8 from Oil Mill Spillage and Enhancement of Lipase Production

Habib M. Thabet[1*] , Chand Pasha [2], Md Maqsood Ahmed[2] and Venkateswar R. Linga[2]

[1]*Department of Food Science and Technology, IBB University, IBB, P.O.Box 70270,Yemen,*

[2] *Department of Microbiology, Osmania University, Hyderabad-500 007, India*

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Abstract

In this study a total of 26 microorganisms were isolated for lipase production. Yeast strain possessing the highest lipase activity was identified as *Sporobolomyces salmonicolor*, and subjected to mutagenesis to increase the enzyme production. Effect of pH, temperature and medium composition on lipase production was studied. *Sporobolomyces salmonicolor* a novel yeast was isolated from oil spillage and the production conditions were optimized. The lipase activity was increased by mutagenesis and with the optimum conditions at pH 6 and 28 ^{o}C for 96 h. The mutant yeast strain OVS8 was selected as it showed stability and maximum lipase production, which is 3.2 times greater than the wild strain (38.5±0.21U ml^{-1}). A novel lipase producing yeast was isolated from oil spillage and the lipase activity was enhanced. Use of *Sporobolomyces salmonicolor* OVS8 for lipase production may be advantageous over Bacteria and *Candida.*

Keywords: Lipase, *Sporobolomyces Salmonicolor*, Ethidium Bromide, Oil Spillage, Tributyrin

1. Introduction

Lipases (EC 3.1.1.3; triacylglycerol lipases) catalyze the hydrolysis and synthesis of long-chain acylglycerols with triolein as the standard substrate (Josana *et al.,* 2009). lipases can catalyze esterification, interesterification and transesterification reactions in non-aqueous media, microbial lipases are biocatalysts that have interesting characteristics, as action under mild conditions, stability in organic solvents, high substrate specificity and regio- and enantioselectivity (Snellman *et al.,* 2002). Though many microorganisms secrete lipolytic enzymes; *Candida*, *Pseudomonas* and *Rhizopus* sp. are the most commonly used for industrial lipase production (Pandey *et al.,* 1999). Since each industrial application requires specific properties of the enzyme, there is a need for finding new lipases that could have novel applications (Jaeger *et al.,*1994; Pandey *et al.,* 1999; Jeganathan *et al.,* 2006).

Research and development studies are needed for strain improvement and medium optimization to increase the lipase production, as the enzyme produced by the wild strain is usually low. Montesinos *et al.* (1997) reported that the fermentation of lipase from *Candida rugosa* and showed 20-40 U ml^{-1} enzyme production. Benjamin and Pandey (1996) optimized the media for lipase production using *C. rugosa* with the maximum lipase activity being 12.55U ml^{-1}. Moreover, no lipase production was observed in the absence of olive oil since olive oil seems to work as an inducer for the excretion of lipase (Liu CH *et al.,* 2006). Kaushik *et al*., (2011) who pointed out that the most important factor was the oil substrate (corn oil). Oil plant wastes make an important source for the isolation of new lipolytic microorganisms. Spontaneous mutations occur at a low rate, and therefore yeast cells are often treated with mutagens to increase the frequency of mutants. Mutant strains of yeast can be obtained by treating yeast with ultraviolet (UV) light, ethyl methanesulfonate (EMS) and or N-methyl-N0-nitro-N-nitrosoguanidine (NTG) as chemical mutagens (Winston, 2008). In this work, we attempted to isolate a novel yeast strain that produce lipase and to enhance the lipase production the yeast was subjected to UV irradiation and ethidium bromide mutagenesis, optimization of the media and cultural conditions.

2. Materials and Methods

2.1. Isolation of lipolytic yeast

Lipase producing microorganisms were isolated from an oil waste site collected from the fortune sunflower oil plant located in Hyderabad (India). The isolation process

* Corresponding author. e-mail: habeeeb3@yahoo.com,habeeb30@gmail.com.

was carried out by serial dilution of the sample and plating on tributyrin selective medium (peptone 5g l^{-1}; beef extract 3g l^{-1}; tributyrin 15ml l^{-1} and agar 20g l^{-1}) plates. The medium was supplemented with 50mg l^{-1} ampicillin and 50mg streptomycin for inhibition of bacterial growth. Colonies were observed on the selective medium after 72 h and colonies, which were showing a clear zone, were selected. Isolates were purified on tributyrin agar plates and transferred to YM agar slants (10g l^{-1} glucose; 3g l^{-1} yeast extract; 5g l^{-1} malt extract; 5g l^{-1} peptone and 20g l^{-1} agar at pH 6.2. The pure cultures were maintained on YM agar medium at +4 ^{o}C and transferred onto fresh medium after 8 weeks.

2.2. Microorganism identification and mutagenesis

2.2.1. Microorganism identification

Isolated yeast was characterized by growth and microscopic morphology and based on gas chromatography of cellular fatty acids using GC-FAME method.

2.2.2. UV mutation

A wild *S. salmonicolor* was grown for 36 h, cells were washed with distilled water and re-suspended in sterilized phosphate buffer solution (pH 7 0.1 mol^{-1}) in order to achieve cell density of 1 $\times 10^{6}$ cells per ml .Two ml of the above cell suspension was placed on sterile petri dish and exposed to UV rays (235nm, 30 Watt) at distance of 15 cm for 20-100 seconds. After UV exposure, 0.1 ml of cell suspension was taken, diluted, plated on sterile tributyrin agar plates and incubated at 30 ^{o}C for three days. Eleven colonies were selected on selective media based on hydrolysis zone and growth on tributyrin agar. These colonies were grown in YM broth at 30 ^{o}C for 36 h at 150 rpm. Five percent inoculum of these cultures was used for fermentation studies.

2.2.3. Mutation by incorporation of mutagen in the medium

Two UV mutants (UV40, UV70) showing maximum lipase production were selected and grown on selective media (Tributyrin agar) in which Ethidium bromide was incorporated at different concentrations as explained by Pasha *et al.,* (2005) and Pasha *et al.,* (2007). Yeast was inoculated on eight plates containing various concentrations of Ethidium bromide (10-30 µg ml^{-1}) and incubated at 30 ^{o}C.

2.3. Fermentation medium

Glucose 20 g l^{-1}; corn steep liquor 10 g l^{-1}; yeast extract 5 g l^{-1}; Tween 80 5ml l^{-1}; K_2PO_4 3 g l^{-1}; $MgSO_4.7H_2O$, 0.5 g l^{-1} ; olive oil 10 ml l^{-1} and pH 6.2 was used for the submerged fermentation. The fermentation was carried out using 50 ml medium with 5 % (v/v) inoculum in 250 ElrenMayer flask at 30 ^{o}C , 150 rpm, by maintaing pH 6.0 for 120 h. Samples were collected every 12 h and used for estimation of lipase activity.

2.4. Lipase assay

Lipase activity was determined using a spectrophotometer and with the *p*-nitro phenyl palmitate (*p*-NPP) as substrate as reported by Gupt *et al.,* (2002). The substrate solution was prepared by adding 30 mg of p-NPP to 10 ml of isopropanol with stirring to dissolve the substrate. This was added to 90 ml of 50 mM Tris–HCl buffer (pH 8). The mixture of 2.4ml of substrate solution and 0.1ml of appropriate diluted enzyme solution was incubated at 40±1 ^{o}C for 10 min and absorbance was measured at 410 nm in a spectrophotometer (Systronic UV Vis Spectrophotomter 117-Ahmedabad, India). One unit of lipase activity was defined as the amount of enzyme required to release 1 µmole of p-NP per ml per minute at 40 °C and pH 8.

2.5. Stability of mutant and optimization of cultural conditions and medium components for lipase production

S. salmonicolor OVS8 was transferred from one YM agar slant to the second slant and was incubated at 30 ^{o}C for 36 h. Yeast from the second slant was transferred to the third slant, and the third slant was subcultured under the same condition for many times. The yeast culture from each generation was transferred to 50 ml fermentation medium in 250 ml flask for fermentation. Samples were collected and checked for lipase activity using spectrophotometry. To enhance the lipase production using developed mutant strain *S. salmonicolor* OVS8, attempts were made to optimize cultural conditions and media components by changing one variable at a time.

2.5.1. Effect of pH and temperature on the lipase production

The yeast strain was grown in fermentation medium for 96 h at 30 ^{o}C at different pH ranges. Medium was prepared with phosphate buffer of various pH (4-9). The yeast strain was cultivated in fermentation medium for 96 h at different temperature (26-34 ^{o}C). The cell free supernatant obtained after centrifugation at 8000 rpm was used for lipase assay.

2.5.2. Effect of various carbon and nitrogen sources on the lipase production

Effect of different carbon sources on lipase production was evaluated by growing the yeast strain in the medium containing various carbon sources (glucose, glycerol, sucrose, maltose, lactose, starch and fructose) at 2% concentration as sole carbon source. The effect of various nitrogen sources on lipase production was studied by growing the yeast strain in the medium containing various nitrogen sources (yeast extract, urea, peptone, beef extract, soybean meal, casein, CH_3COONH_4, NH_4Cl, (NH_4) NO_3 and $(NH_4)_2SO_4$ at 1 % concentration as sole nitrogen source.

2.5.3. Effect of various lipids as inducers and metal ions on lipase production

The effect of various lipids as inducers on lipase production was evaluated by growing the yeast strain in the medium containing various lipids and fatty acids such as groundnut oil, sesame oil, mustard oil, tributyrin, soybean oil, oleic acid, olive oil, sunflower oil, almond oil and coconut oil at 0.3% concentration. The yeast strain was grown in fermentation medium containing various metal ions ($MgSO_4$, $MnSO_4$, $CaCl_2$, K_2HPO_4, NaCl, $ZnSO_4$, Na_2HPO_4 and $FeSO_4$) for 96 h at 30 ^{o}C at 0.1% concentration. The cell free supernatant was used for lipases assays. All the experiments were performed in triplicates and the average mean values are reported.

3. Results

3.1. Isolation and identification of lipolytic yeast

Microorganisms showing lipolytic activity were isolated from oil spillage of sunflower oil factory. Seven bacterial and 19 yeast strains were isolated from selected oil waste samples (Table 1). Lipase-producing microorganisms were selected after screening on selective tributyrin agar with the formation of clear hydrolysis zone because of extracellular lipase activity. Three bacteria and four yeast isolates producing high amounts of lipase were selected based on colony to hydrolysis zone ratio. All the isolates obtained in screening were used for lipase production in liquid medium. The lipase produced by yeast (8.2±1.40U ml^{-1}) was more than the bacteria 2.1±0.77U ml^{-1}.

Table 1. Production of lipolytic enzyme by bacteria and yeast isolates

S.No	Strain No	Growth colony diameter (mm)	Activity zone diameter (mm)	Ratio of colony /zone (mm)	Enzyme activity (U/ml) mean+SD
1	BM-1	5	4	1.2	0.72±0.21
2	BM-2	5	3	1.6	0.60±0.30
3	**BM-3**	**3**	**6**	**0.5**	**2.1±0.77**
4	BM-4	7	4	1.7	0.9±0.42
5	**BM-5**	**5**	**8**	**0.6**	**1.6±0.83**
6	**BM-6**	**7**	**9**	**0.7**	**1.2±0.54**
7	BM-7	9	2	4.5	0.4±0.18
8	OMV-1	7	9	0.7	5.5±1.40
9	OMV-2	9	7	1.2	6.0±1.10
10	OMV-3	7	8	0.8	6.2±1.30
11	OMV-4	6	9	0.6	6.8±1.82
12	OMV-5	6	9	0.6	6.0±1.10
13	OMV-6	8	6	1.3	5.7±0.68
14	**OMV-7**	**6**	**10**	**0.6**	**7.6±1.50**
15	OMV-8	9	7	1.2	3.0±0.35
16	OMV-9	9	8	1.1	5.4±0.82
17	OMV-10	7	5	1.4	3.2±0.40
18	OMV-11	9	8	1.1	6.0±1.20
19	OMV-12	8	6	1.3	4.6±0.95
20	**OMV-13**	**7**	**11**	**0.6**	**8.0±1.40**
21	**OMV-14**	**6**	**9**	**0.6**	7.5±1.10
22	**OMV-15**	**5**	**12**	**0.4**	**8.2±1.40**
23	OMV-16	9	8	1.1	6.2±1.00
24	OMV-17	9	7	1.2	4.0±0.65
25	OMV-18	6	8	0.7	5.6±1.20
26	OMV-19	8	6	1.3	3.4±0.63

3.2. Identification

The yeast strain isolated with highest lipase production from sunflower oil mill waste was used for identification. Colony morphology of yeast strain showed white color, round and small size (Fig 1). The isolated yeast when observed under the microscope without staining the cells was simple filamentous to elaborate pseudohyphae. Budding and exopolysaccharide coated sheath was observed around the cells which is also a typical property of *Sporidiobolus* sp (Fig 2). Yeast strain was also identified as *Sporobolomyces salmonicolor* using Microbial (MIDI microbial identification system chenni, India).

Figure.1 *S.salmonicolor* colonies as seen on MY agar

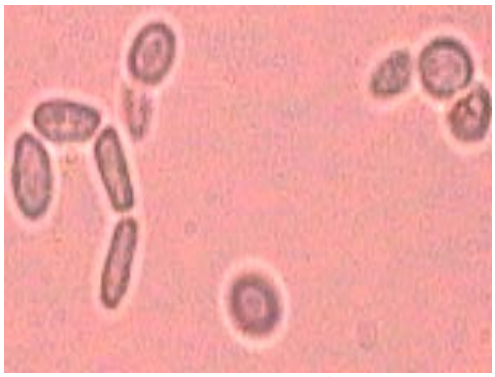

Figure. 2 Microscopic examination of *S. salmonicolor*

3.3. Selection of lipolytic mutant yeast

Based on lipolytic activity and colony morphology, mutants were selected. The UV mutant yeast strains were screened by growing on tributyrin agar. Two highest lipase producing mutant yeast strains were selected and subjected to ethidium bromide mutation.

The UV treatment on *S.salmonicolor* for more than 100 second killed more than 90% cells. Eight strains with more than 17 U ml^{-1} lipase activity was initially selected, and based on stability the mutant yeast strains (UV40, UV70) with lipase activity 18.2±1.20 and 18.9 ±1.10 U ml^{-1} respectively were finally selected for further experiments. The influence of UV radiation time is shown in (Table 2).

Two UV mutant yeast strains (UV40, UV70) were further subjected to mutation with ethidium bromide and the results are presented in (Table 3). The yeast growth was observed in the control within 36 h, and the growth in other mutated plate was observed after five days of incubation. Six colonies having less colony to hydrolysis

zone ratio were selected for lipase production. When the concentration of mutagen was higher than 20 µg ml^{-1} the survival of cells was less than 3%. Four strains having lipase activity more than 31.5±1.50 U ml^{-1} were initially selected. After optimization of medium composition, the mutant *S. salmonicolor* OVS8 yielded 38.5±0.21 U ml^{-1} of lipase. Mutant Strain OVS8 showed highest lipase activity, and it was selected for further experiments to optimize cultural conditions and media components for the increased lipase production.

Table 2. UV mutants and their activity

UV mutant	lipase activity (U ml^{-1})
UV10	17.5± 0.90
UV20	17.0± 1.10
UV30	17.6± 0.85
UV40	18.2± 1.20
UV50	17.8± 0.82
UV60	17.2± 1.30
UV70	18.9 ±1.10
UV80	18.0±1.00

Table 3. Ethidium bromide mutants and their lipase activity

Eth bromide mutant	lipase activity (U ml^{-1})
OVS5	30.5±1.20
OVS6	31.2±1.50
OVS7	31.0±1.10
OVS8	31.5±1.50

3.4. Stability of the mutant and optimization of cultural conditions and media components for lipase production

The stability of selected mutant was tested for 28 generations and *S. salmonicolor* OVS8 was found to be stable with lipase activity 37±1.5 U ml^{-1} (Table 4).

Table 4. Stability of *S. salmonicolor* OVS8

Generation	3	8	13	18	23	28
Lipase activity (U ml^{-1})	37.6± 1.1	39± 2.1	36.2± 1.4	37± 1.5	38± 2.8	37.5± 1.5

Optimization of cultural conditions and media components for production using mutant strain OVS8 was carried out by changing pH, temperature, nitrogen sources, inducers and carbon sources each at one time, in the production medium.

3.4.1. The effect of initial pH and temperature

The extracellular lipase production was influenced by the initial pH of the medium (Fig 3) the optimum pH for lipase production was found to be 6. The effect of temperature on lipase production is shown in (Fig 4).

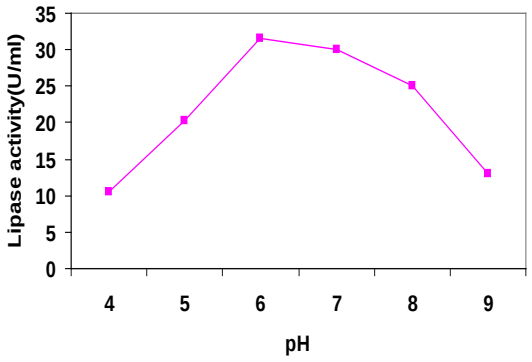

Figure 3. Effect of pH on the lipase production

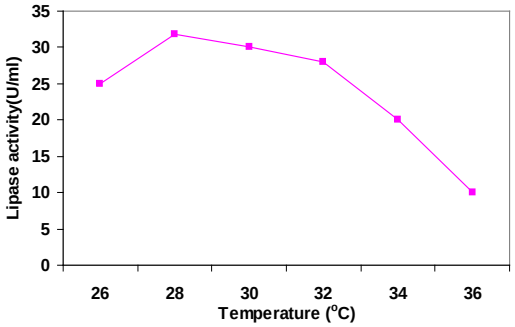

Figure 4. Effect of temperature on lipase production

3.4.2. The effect of carbon source

The study of lipase production with various carbon sources at 2% concentration in the medium showed that the enzyme production was more when glucose (36± 0.24 U ml^{-1}) and lactose (34±0.14 U ml^{-1}) were used (Table 5). Mutant *S. salmonicolor* could utilize several carbohydrates but showed poor growth and low enzyme production with starch and glycerol.

Table 5. Effect of carbon source on lipase production by *S. salmonicolor*

Carbon source Concentration (2%)	Biomass (g l^{-1})	Lipase activity (U ml^{-1})
Glucose	10.6	36.0± 0.24
Glycerol	7.3	19.0±0.23
Sucrose	10.2	30.2±0.29
Maltose	9.3	22.0±0.28
Lactose	10.4	34.0±0.14
Starch	7.2	18.0±0.18
Fructose	8.5	24.0±0.21

3.4.3. Effect of various lipids as inducers on the lipases production

Among different lipids tested, olive oil and sunflower oil were found to be best inducers for lipase production (Table 6) and low activity was obtained with tributyrin. Though the oleic acid supported good growth but very less lipase was produced.

Table 6. Effect of lipids as inducers on lipase production with *S. salmonicolor*

Inducer sources (0.3%)	Biomass (g l^{-1})	Lipase activity (U ml^{-1})
Groundnut oil	9.4	31.5±0.12
Mustard oil	9.2	28.4±0.17
Soybean oil	9.7	34.5±0.21
Olive oil	9.8	38.0±0.26
Sunflower oil	9.4	36.5±0.29
Almond oil	7.8	27.0±0.16
Sesame oil	8.6	20.5±0.22
Tributyrin	7.8	18.5±0.11
Oleic acids	6.6	05.0±0.22
Sunflower oil	9.4	36.5±0.14
Coconut oil	9.0	33.6±0.20

3.4.4. Effect of nitrogen sources on the lipases production

The mutant *S. salmonicolor* has grown well with all the nitrogen sources tested. Yeast extract and ammonium sulphate were the best among all the nitrogen sources tested, giving 37.5±0.57and 37.0±0.92 U ml^{-1} lipase activity (Table 7). The lipase production was low with Beef extract and casein; whereas other inorganic nitrogen sources produced more than 18.5±1.00 U ml^{-1} of enzyme. We have analyzed the data using one way ANOVA with the help of SPSS software, i.e. effect of carbon, nitrogen sources and inducers on the production of lipase and biomass. Results showed that lipase production is significant at 0.05% level between different carbon, nitrogen sources and inducers.

Table 7. Effect of nitrogen sources on lipase production with *S. salmonicolor*

Organic nitrogen (1%)	Biomass (g l^{-1})	Lipase activity (U ml^{-})	Inorganic nitrogen (1%)	Biomass (g l^{-1})	Lipase activity (U ml^{-})
Yeast extract	9.6	37.5± 0.57	Urea	8.4	34.5± 0.41
Peptone	9.5	33.0± 0.89	CH_3COONH_4	7.5	18.5± 1.00
Casein	8.2	18.5± 0.92	NH_4Cl	7.7	24.0± 0.26
Soybean meal	9.4	30.0± 0.50	$(NH_4)\ NO_3$	7.8	22.0± 0.30
Beef extract	7.5	19.0± 0.96	$(NH_4)_2SO_4$	8.0	37.0± 0.92

3.4.5. Effect of metal ions on the lipases production

The effect of metal ions on the lipase production by addition of MgSO4.7H2O and K_2HPO_4 increased the lipase production while $MnSO_4$ and $CuSO_4$ decreased the lipase production on mutant *S. salmonicolor* (Table 8)**.**

For all the experiments the inoculum was grown in YM medium and was performed in triplicate.

Table 8. Effect of metal ions on lipase production with *S. salmonicolor*

Metal ions (0.1%)	Mg SO_4	Mn SO_4	Ca Cl_2	K_2 HPO_4	Na Cl	Zn SO_4	Na_2 HPO_4	Fe SO4
Lipase activity (U ml^{-})	36± 2.1	26± 2.0	22± 1.8	36± 2.5	30± 2.2	18± 1.3	26± 2.4	17± 1.5

4. Discussion

Microorganisms showing lipase activity were isolated from oil spillage of sunflower oil factory. From 26 isolates, one of the highest lipase producing yeast (on tributyrin agar media) was selected. Ertugrul *et al.* (2007) isolated 17 strains from olive oil mill waste water (OMW), using tributyrin agar media and the highest lipase producing microorganism was identified as *Bacillus* sp. Cellular fatty acid analysis is widely used for characterization many microbial species (Moore *et al.,* 1994). The isolated yeast was characterized using this method and identified as *Sporobolomyces salmonicolor*.

In the present study the enhancement of lipase production by yeast was carried out with UV and ethidium bromide methods. Ethidium bromide (12.6 U ml^{-1}) was found to be more efficient in inducing the mutagenesis when compared to UV (6.9 U ml^{-1}). Other mutagenesis reported, the enhancement of lipase production from *Aspergillus niger* was attempted by ultraviolet (UV) and nitrous acid mutagenesis, and one of the mutant strains NAII exhibited 2.53 times more increased lipase activity than the parental strain (Mala *et al.,* 2001). *Y. lipolytica* DSM3286 was subjected to mutation using ethyl methanesulfonate (EMS) and ultraviolet (UV) light. One UV mutant (U6) produced 356 U/mL of lipase after 24 h, which is about 10.5-fold higher than that produced by the wild type strain (Farshad *et al.,* 2011). Using *S. salmonicolor* the optimum found to be 28 ^oC and pH 6 for maximum lipase production. This was supported by Chien-HL, *et al.* (2012) the maximum lipase production was 22.67 U ml^{-1} when the pH of the medium was controlled at 6.0. Dalmau *et al.* (2000) obtained the highest yield of lipase by *C.rugosa* using lipids or fatty acids as carbon sources and also with the mixture of triglycerides and carbohydrates. In the present study the highest lipase activity was found with glucose and lactose, the results are similar to the results of (Costas *et al.,* 2004).

Domínguez *et al.,* (2003) reported that the titer of lipase production was 16.7 U/ml under the optimal conditions with yeast extract of 2.175 g $l^{-1}$and olive oil of 5.54U ml^{-1}. Similar in this study highest lipase activity has obtained by using olive oil (38.0±0.26 U ml^{-1}) correlated with increased biomass (9.8 g l^{-1}). Yeast extract and ammonium sulphate (37.5±0.57 and 37.0±0.92 U ml^{-1}) are the best source of nitrogen for lipase production. Chien *et al.,* (2012) reported the titer of lipase production by *Burkholderia* sp was 16.7 U ml^{-1}under the optimal conditions with yeast extract of 2.175 g l^{-1} and olive oil of 5.54 ml l^{-1}. The metal ions like Mg^{+2} and K^{+} were found to enhance the production of lipase on mutant *S. salmonicolor* (36±2.1 and 36±2.5 U ml^{-1}) respectively. Similar reported by (Tan *et al.,* 2003) reported the highest lipase production by *Candida sp* in the presence of Mg^{+2},

Na^+ and K^+ metal ions. Overall, in this study a novel producing lipase yeast strain has been isolated. Further purification and characteristics of this enzyme may be used in industrial or medical field.

5. Conclusion

A strain of *S. salmonicolor* was isolated and identified using gas chromatography of methyl esters of cellular fatty acids GC-FAME (MIDI). It was selected for further studies as it produced considerable quantities of extracellular lipolytic enzyme. The yeast strain was further improved using UV and Ethidium Bromide which lead to enhanced lipase production (38.5±0.21U ml^{-1}).

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