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Optimization and characterization of a thermo tolerant lipase from *Cryptococcus albidus*

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ABSTRACT

A thermo tolerant yeast Cryptococcus albidus was characterized for optimal production of liapse . The organism shown maximum production on 5th day of incubation. The optimum temperature for both production and activity was 50^{0}C and optimal pH was 6.5. Few metal ions like Ba^{2+}, Ca^{2+}, Mg^{2+} and Zn^{2+} enhanced the activity whereas Fe^{3+}, Mn^{2+} and Zn^{2+} declined the enzyme action. The enzyme was characterized by some inhibitors and solvents; amongst inhibitors, β – mercapto ethanol and SDS had shown maximum inhibition where as amongst the solvents phenol and choloroform strongly repressed the activity. Different solid and liquid raw materials viz; oil cake, vegetable oils and ghee extraction waste were also studied for enzyme production.

Keywords: *Cryptococcus albidus*, lipase, enzyme activity, production, relative activity.

INTRODUCTION

Lipases (EC 3.1.1.3) catalyze the hydrolysis of acyl glycerols to fatty acids, di-acyl glycerols, mono-acyl glycerols and glycerol and widely occur in bacteria [1,2,3], yeasts and fungi [4,5]. Among microbes, fungi including yeasts are extensively recognized as the best lipase sources and are used preferably for industrial applications because fungal enzymes are usually excreted extracellularly, facilitating extraction from the fermentation media. A large number of unicellular as well as filamentous fungi have been studied for lipase production [5,6]. *Aspergillus*, *Rhizopus* and some strains of yeast are well known lipase producer and suitable for use in many industrial applications [7,8] . Studies on conditions for the production of extra- cellular lipases by fungi show variations among different strains but the requirement of lipid carbon source is crucial for enzyme production. Hence, the technique of solid state fermentation (SSF) is exploited at industrial scale and involves the growth and metabolism of microorganisms on moist solids. This

technique has many advantages *viz*. economy of space needed for fermentation, simplicity of fermentation media, less energy demand, lower capital and recurring expenditure [9,10]. Although, almost all literature on SSF refers to fungal systems but there are very few reports on lipase production in SSF by yeast to date [11,12]. Lipases active at highly acidic pH have not been reported so far from microbial sources. Therefore, the present study is standardized for lipase production by a selected yeast stain *Cryptococcus albidus* and characterization of the enzyme with regard to thermo stability, pH stability and optimum temperature and pH conditions for reaction.

MATERIALS AND METHODS

Isolation of fungi from soil

A high lipolytic yeast *Cryptococcus albidus* was isolated from oil rich samples collected from different niches of Rajasthan. The pure culture of yeast was maintained on GYPA medium at 28±1 °C whereas one set of culture was maintained at 4 °C in refrigerator for further use.

Growth medium and cultural conditions

1 ml of olive oil along with 100 ml basal salt solution (Peptone: 0.5 g; $MgSO_4.7H_2O$: 0.05 g; KCl: 0.05 g; KH_2PO_4: 0.2 g; $NaNO_3$: 0.05 g) in 250 ml of Erlenmeyer flask were autoclaved at 15 psi for 15 minutes. These flasks were inoculated with *Cryptococcus albidus* and incubated at 28±1 °C in shaking incubator (80 rpm) for 5 days.

Enzyme assay

Crude enzyme extract was prepared from the culture supernatant which was centrifuged at 10,000 rpm for 15 min at 4˚C. Lipase activity was measured spectrophotometrically using p-nitrophenyl acetate (pNPA) as a substrate at 45°C in 100 mM phosphate buffer of pH 7.0[13]. The substrate for this reaction was composed of solution A and B. Solution A contained 40 mg of p-nitro phenyl actetate dissolved in 12 ml of isopropanol, solution B contained 0.1 g of gum arabic and 0.4 ml of triton X-100 dissolved in 90 ml of distilled water. The substrate solution was prepared by adding 1 ml of solution A and 19 ml of solution B. The assay mixture contained 1 ml of the substrate, 0.5 ml of buffer, 0.1 ml of enzyme and final volume was made up to 3 ml with distilled water. The enzyme activity was stopped by adding 0.2 ml isopropanol and liberation of *p*-nitrophenol at 28 °C was detected in spectrophotometer at 400 nm. One enzyme unit was defined as 1 µmol of p-nitrophenol enzymatically released from the substrate per minute [1].

Effect of various parameters on lipase activity

Effect of incubation period

Enzyme production was observed from 1^{st} to 10^{th} days of incubation and the activity was determined by similar protocol as described above. 0.1 ml of crude enzyme extract added in substrate solution, incubated at 28 °C for 30 minutes and lipase activity was measured spectrophotometrically against control.

Effect of temperature

The optimum temperature of the enzyme were determined with p-nitro phenyl acetate by incubating the assay mixture in the temperature range of 10°C to 90°C using phosphate buffer

pH 7 (0.5 M). To ascertain the stability, 0.5 ml of phosphate buffer (0.5M, pH 7) and 0.1 ml of enzyme were incubated at different temperatures (10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C and 70 °C) for 30 minutes and assayed for lipase activity.

Effect of pH on lipase activity and stability

The titrimetric assay of lipase produced by the test strain was performed according to the method of Kamimura *et al.* [14]. Lipase activity was measured with 0.05 M NaOH using emulsified olive oil as the substrate. One ml of culture supernatant, treated as crude enzyme was added to 5 ml of oil emulsion containing 25 % (v/v) olive oil, 75 % (v/v) gum arabic and 2 ml of 0.5 M phosphate buffer at pH 7.0. The assay was carried out at 37 ^{0}C during 30 min incubation. After this time interval the reaction was stopped by addition of 15 ml of acetone/ ethanol (1:1 v/v) and the amount of fatty acids was then titrated. 1 ml of the titration volume is equal to 2.5 units of lipase.
For determination of stability on distinct pH, the buffers used were glycine-HCl buffer (pH 3.5), citrate buffer (pH 3.5-5.0), phosphate buffer (pH 5.5-7.5), tris-HCl buffer (8.0-8.5) and glycine-NaOH buffer (pH 9.0- 12.0). 0.1 mL of enzyme was incubated with different buffers along with substrate solution at 28°C for 30 minutes and assayed for lipase activity.

Effect of metal ions on lipase activity

For determining the effect of different metal ions on lipase activity were incubated with two different concentrations *i.e.* 50 µg/ml and 100 µg/ml of Mn^{+2} ($MnCl_2$), Cu^{+2} ($CuSO_4$), Zn^{+2} ($ZnSO_4$), Ba^{2+}($BaCl_2.5H_2O$), Fe^{+2} ($FeCl_3$), Hg^{+2} ($HgCl_2$), Ca^{+2} ($CaCl_2.6H_2O$), Co^{+2} ($CoCl_2.6H_2O$), Mg^{+2} ($MgCl_2.6H_2O$), NH_4^+ (NH_4NO_3 anhy.) and Ag^+ ($AgNO_3$) for 30 min at 50 °C under standard assay conditions.

Effect of inhibitors on lipase activity

The effect of different inhibitors on lipase activity were determined by mixing with two distinct concentrations (50 µg and 100 µg per ml of reaction mixture). Following chemical inhibitors *viz*; Urea, H_2O_2, sodium nitrite, sodium hypochlorite, sodium bisulphate, EDTA, DTT, SDS, β-mercapto ethanol, DMF and PMSF were used and activity was evaluated at 50 °C for 30 min (pH 6.5) under standard assay conditions.

Effect of solvents on lipase activity

The effect of different solvents on lipase activity was determined by incubating the reaction mixture with 50 µl/ml and 100 µl/ml concentrations of acetone, aniline, benzene, chloroform, ether, iso propanol, ethanol, glycerol, phenol, titran X- 100 and toluene individually for 30 min at 50 °C under standard assay conditions.

Effect of different edible oils on enzyme production

The present experiment was performed as per the protocol developed by Lima *et al.* [15] in order to determine the best carbon source for the test strain and lipid degrading capability, on various vegetable oils. For this purpose 1 % each of olive oil, coconut oil, ground nut oil, mustard oil, soy bean oil and ghee was added separately to 50 ml of the basal medium. The contents were incubated at 28±1 ^{0}C and 5th day culture broth of *Cryptococcus albidus* was taken as crude extract enzyme.

Lipase production from raw materials like oil cakes and ghee extraction waste:
The experiment was performed as per Lima *et al*. [15]. 10 ml of substrate moistening reagent (100 mM of phosphate buffer, pH 7) along with 2 g of crushed oil cake / 2 g of ghee extraction waste and sterilized. 2 ml of strain inoculum was inoculated and incubated at 28±1 ^{0}C. Lipase activity was observed from 1st to 10th day of incubation. The enzyme was procured by taking 25 ml of chilled phosphate buffer of pH 7 was added in each flask and mixed properly. Crude enzyme extract was filtered through filter paper and lipase activity was determined as described earlier.

RESULTS AND DISCUSSION

From the Table-1, it is revealed that the production of enzyme increased during cell growth of *Cryptococcus albidus* and reached maximum (47.2 U/ml) on 5th day of incubation in batch culture, after that the production was decreased with increase in incubation period. Gumienna *et al*. [16] reported similar observation while studying the lipase production from *Candida bombicola* ATCC 22214. In contrary to this Kim *et al*. [4] reported optimum production from *Pichia lynferdii* on 4 days of incubation. The optimum temperature for lipase production by this organism was determined and tested at the range of 20 to 60 ˚C (Table -2). It was observed that 50 ˚C was the optimum incubation temperature. This shows that the lipase produced by this organism is thermo tolerant. Therefore, this enzyme can be exploited for detergent and food processing industries. Imandi and Garapati [17] reported maximum yield of enzyme incubating the organism at 30 ^{0}C while studying the lipase production from *Yarrowia lipolytica* NCIM 3589.

Activity of lipase, produced by test strain, using different lipid substrates was determined using *p*-NPP as substrate. In substrate buffer, instead of *P*-NPP, various lipid sources were used in appropriate concentration. Lipase assay was done under standard condition and relative activity was measured.

Table 1: Effect of incubation period on lipase production by *Cryptococcus albidus*

Incubation Period (Days)										
Lipase activity (U/ml) (Each value is an average of three parallel experiments)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
	19.7	21.3	27.8	33.5	47.2	47.1	38.5	31.5	29.3	29.1

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Effect of pH and temperature

The pH plays crucial role for the detection of enzymatic activity. The study of the effect of pH on enzymatic activity and stability provides valuable clues regarding the type and identity of amino acids present in the enzyme. Enzymes are affected by changes in pH and show maximum activity at a specified pH. Extremely high or low pH values generally result in complete loss of activity for most of enzyme. In this study, highest activity at pH 6.5 was recorded (Table-3) and as the pH increased beyond 6.5, the activity was dropped. Korbekandi *et al*. [18] and Kim *et al*. [4] both reported optimum pH 7 while working on *Candida rugosa* and *Pichia lynferdii* respectively.

The crude enzyme shown maximum activity and stability at 50 ^{0}C and retained 57 % of its activity at 60^{0}C, indicating thermo tolerant nature of the enzyme (Table- 4). Similar observation

was recorded by Deive *et al*. [19] and Jatta *et al*. [20] while working on optimum lipase production from *Kluyveromyces marxianus* and *Candida albicans* respectively.

Table 2: Effect of temperature on lipase production by *Cryptococcus albidus*

Temperature (^{0}C)						
Lipase activity (U/ml)	**20**	**28**	**35**	**40**	**50**	**60**
(Each value is an average of three parallel experiments)	23.4	30.5	27.0	31.7	44.9	34.5

Table 3: Effect of pH on lipase activity and stability of *Cryptococcus albidus*

	pH													
	3.5	**4.0**	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**	**9.0**	**10**	**11**	**12**
RA (%)	21.45	32.86	48.63	70.68	76.15	84.68	100	82.45	61.42	49.66	42.56	35.65	31.23	19.65
RS (%)	27.75	31.45	44.78	67.83	72.46	81.23	100	79.30	58.65	43.54	41.85	32.48	29.87	18.68

RA = Relative activity; RS = Relative stability (Each value is an average of three parallel experiments)

Table 4: Effect of temperature on lipase activity and stability of *Cryptococcus albidus*

	Temperature (^{0}C)										
	5	**15**	**25**	**30**	**35**	**40**	**50**	**60**	**70**	**80**	**90**
Relative activity (%)	25.76	21.65	30.13	43.67	51.53	52.84	100	57.35	34.97	38.03	32.31
Relative stability (%)	25.35	26.45	29.56	44.76	52.40	53.27	100	57.38	45.89	36.57	30.47

Effect of various metal ions, inhibitors and solvents

Certain enzymes besides coenzymes require a metal ion for their full activity. As reported from studies on this isolate at the concentration of 50 µg/ml of some metal ions could affect the enzyme activity. Ba^{2+}, Ca^{+2} and Mg^{2+} and NH_4^+ enhanced the activity of enzyme. Whereas metal ions Fe^{+3}, Mn^{+2}, Zn^{+2}, Co^{+2}, Cu^{+2} and Hg^{+2} showed above 60-70 % relative activity, on increasing the concentration up to 100 µg/ml, all the ions inhibited the enzyme activity except Ag^+ (Table- 5). Hg^{+2} showed the stronger inhibitory effect and showed 36.7 % relative activity. Kakugawa *et al*. [21] characterizing lipase of *Kurtzmanomyces* sp. reported 19 % and 46 % relative activity when added Fe^{+2} and Al^{+3} at the concentration of 50 µg/ml in the reaction mixture.

Table 5: Effect of different metal ions on lipase activity of *Cryptococcus albidus*

		Metal ions										
		Control	Mn^{2+}	Cu^{2+}	Zn^{2+}	Ba^{2+}	Fe^{2+}	Hg^{2+}	Ca^{2+}	Co^{2+}	Mg^{2+}	NH_4^+	Ag^+
Relative activity (%)	**Solvent (50 mM)**	100	74.5	66.9	73.9	111.7	58.5	44.1	135.6	91.8	137.9	129.2	32.8
	Solvent (100 mM)	100	67.4	61.6	69.9	107.9	54.3	36.7	128.6	86.3	123.5	117.6	32.8

A total of eleven inhibitors were studied with crude enzyme extract at the concentration of 50 and 100 µg/ml individually in enzyme assay. β-mercapto ethanol (0.33 U/ml) strongly inhibited the activity of enzyme followed by SDS (19.8%), other inhibitors showed 20-60% relative activity at 50 µg/ml concentration. On the increasing the concentration all showed strong inhibitory effect (Table- 6). In contrary to this Balaji and Ebenezer [10] reported that SDS strongly reduces the enzyme activity while working on extracellular lipase production from *Colletotrichum gloeosporiodes*. Amongst 10 solvents, phenol and choloform at 50µl/ml concentration, showed greater inhibitory effect but acetone, iso- propanol and ethanol partially

inhibited whereas aniline and benzene enhanced the activity at the same concentration. The enhancement of lipase activity via these solvents have many applications as in oil based fuel manufacturing [22]**,** plastic and chemical manufacturing, biodegradation, pulp and paper industries, dairy and food industries [11]. On the increased concentration of 100 µl/ml, all the solvents dropped the activity as compared to 50 µl/ml (Table-7). Devie *et al*. [19] reported that lipase produced by *Kluyveromyces marxianus* shown 70 % of its residual activity after 2 days in solvent combination of *n*-hexane and cyclohexane (80 % v/v). Kakugawa *et al*. [21] reported stimulation of *Kurtzmanomyces* sp. lipase activity in the presence of 50 % isobutanol, xylene, benzene and toluene however they reported dramatically reduced enzyme activity in the presence of methanol, choloroformand dimethylsulfoxide (DMSO).

Table 6: Effect of different inhibitors on lipase activity of *Cryptococcus albidus*

		Inhibitors											
		Control	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
Relative activity (%)	**Inhibitor (50 mM)**	100	29.2	49.5	48.4	52.7	44.5	49.6	57.3	29.5	0.7	37.9	57.6
	Inhibitor(100 mM)	100	23.7	41.5	41.7	47.9	38.6	40.3	48.9	19.8	0.2	30.5	50.5

Whereas; A= Urea; B=H_2O_2; C= Sodium nitrite; D=Sodium hypochlorite; E=Sodium bisulphate; F=EDTA; G=DTT; H=SDS; I=β-mercapto ethanol; J=DMF and K= PMSF

Effect of different edible oils on enzyme production

Efficacy of different vegetable oils were tested for the enzyme production. Soy bean oil supported maximum enzyme yield (52.5 U/ml) followed by olive oil (49.2 U/ml), coconut oil (47.9 U/ml), ghee (45.4 U/ml), ground nut oil (43.5 U/ml) and mustard oil (41.7) (Table - 8). Imandi and Garapati [17] reported maximum lipase yield with sunflower oil with the same organism.

Table 7: Effect of different solvents on lipase activity of *Cryptococcus albidus*

		Solvents											
		Control	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
Relative activity (%)	**Solvent** (50 µl/ml)	100	79.6	146.8	123.5	41.8	69.8	74.8	71.8	54.7	00.0	43.5	67.1
	Solvent (100 µl/ml)	100	77.8	137.6	119.8	36.2	64.7	69.8	63.9	47.4	00.0	36.7	58.5

Whereas, A = Acetone; B= Aniline; C= Benzene; D= Chloroform; E= Ether; F= Iso propanol; G= Ethanol; H= Glycerol; I = Phenol; J= Titran X - 100 and K= Toluene

Lipase production from raw materials like oil cakes and ghee extraction waste:

The present experiment was an attempt for lipase production through waste materials, like oil cake and ghee extraction waste. These can be an excellent source for lipase production as lipase activity obtained in these sources was very much higher than different oil sources. Ghee extraction waste was the best source obtained for lipase production, and could be a very good alternative for mass scale production of lipase in lowest cost. The organism shown enzyme yield of 68.7 U/ml (Table- 9). Since these source are the cheapest one, and easily available, they could proved to be very good source for industrial scale production of lipase. Whereas when mustard oil cake shown significant activity (49 U/ml) (Table- 9).

Gumienna *et al*. [16] reported maximum lipase production from *Candida bombicola* ATCC 22214 using post-deodorizing condensate in the production media. The present investigation on lipase produced by *Cryptococcus albidus*, showed that yeasts are one of the best producer of lipase in batch culture, which are active at pH 6.5 (acidic lipase) and find application in different medical and many esterification processes [23]. The optimum temperature for the enzyme activity was at 50 ˚C. The thermotolerant lipase are using various industries such as oleochemical, food additives, cosmetics and therapeutics. The high regio and spacio specificities of these enzymes have application in the kinetic resolution of optical isomers for the synthesis of optically pure substances in pharmaceutical and chemical industries [24,25].

Table 8: Effect of different edible oils on lipase production from *Cryptococcus albidus*

Edible oils	Mustard oil	Coconut oil	Ghee	Groundnut oil	Olive oil	Soya bean oil
EA (U/ml)	41.7	47.9	45.4	43.5	49.2	52.5

Table 9: Raw materials as substrate for lipase production from *Cryptococcus albidus*

S. No.	**Raw material**	**Enzyme activity (U/ml)**
1	Ghee extraction waste	68.7
2	Oil cake	49.0

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REFERENCES

[1]. M. N. Syed, S. Iqbal, S. Bano, A. B. Khan , S. Ali-ul-Qader and A. Azhar, *African Journal of Biotechnology*, 9 (45), 7724-7732 (**2010**).
[2]. E. Sirisha, N. Rajasekar and M. L. Narasu, *Advances in Biological Research*, 4 (5), 249-252 (**2010**).
[3]. B Yuan, Y Cai, X Liao, L Yun, F Zhang and D Zhang, *African Journal of Biotechnology*, 9(18), 2661-2667 (**2010**).
[4]. H. R. Kim, I. H. Kim, C. I. Hou, K. I. Kwon and B. S. Shin, *J. Agric. Food Chem*., 58, 1322–1326 (**2010**).
[5]. S. B. Imandi, S. K. Karanam and H. R. Garapati, *Advances in Bioscience and Biotechnology*, 1, 115-121 (**2010**).
[6]. T. Iftikhar, M. Niaz, M. Afzal, I. Ul- Haq and M. I. Rajoka. *Food Technol. Biotechnol*, 46 (4), 402–412 (**2008**).
[7]. P. Shukla, K. Gupta and N. Kango, *Research Journal of Microbiology*. 2 (9), 671-677 (**2007**).
[8]. A. Jayaprakash and P. Ebenezer, *Indian Journal of Science and Technology*, 3 (2), 113-117 (**2010**).
[9]. J. C. Falony, G. Armas, J. C. D. Mendoza and J. L. M. Hernandez, *Food Technol. Biotechnol.*, 44 (2), 235–240 (**2006**).
[10]. V. Balaji and P. Ebenezer, *Indian Journal of Science and Technology*, 1 (7), 1-8 (**2008**).
[11]. A. Pandey, P. Selvakumar, C. R. Soccol and P. Nigam, *Curr. Sci*., 77, 149-162 (**1999**).
[12]. J.F.M. Burkert, R.R. Maldonado, F. M. Filho and M. I. Rodrigues, *Journal of Chemical Technology and Biotechnology*, 80, 61–67 (**2005**).

[13]. M. P. Licia, M. R. Cintia, D. B Mario and R. C. Guillermo, *Food Technol. Biotechnol*., 44(2), 247-252 (**2006**).
[14]. E. S. Kamimura, O. Medieta, M. L. Rodrigues, and F. Maugeri, *Biotechnol.Appl.Biochem.*, 33, 153-159 (**2001**).
[15]. V. M. G. Lima, N. Kriegerb, M. I. M. Sarquis, D. A. Mitchell, L. P. Ramos and J. D. Fontana, *Food Technol. Biotechnol.*, 41 (2), 105-110 (**2003**).
[16]. M. Gumiena, R. Z. Wojtasiak, M. Czarnecka and Z. Czarneki, *Pol. J. Food Nutr. Sci*., 12/53 (3), 15-20 (**2003**).
[17]. S. B. Imandi and H. R. Garapati, *Research Journal of Microbiology*., 2 (1), 88-93 (2007).
[18]. H. Koebekandi, D. Abedi, M. Pourossein, M. M. Bashi, M. Hejari, M. Narimousaei and M. Kabiri, *Biotechnology*., 7 (1), 112-117 (**2008**).
[19]. F. J. Deive, M. Costas and M. A. Longo, *Biot.echnology Letters*., 25, 1403–1406 (**2003**).
[20]. B. Jatta, M. Gunasekaran and N. Mohan, *Asian Journal of Biotechnology*., 1 (3), 118-123 (**2009**).
[21]. K. Kakugawa, M. Shobayashi, O. Suzuki and T. Miyakawa, *Biosci. Biotechnol. Biochem.*, 66 (5), 978-985 (**2002**).
[22]. M. Kaieda, T. Samukawa, T. Matsumoto, K. Ban, A. Kondo, Y. Shimada, H. Noda, F. Nomoto, K. Ohtsuka, E. Izumoto and H. Fukuda, *J. of Bioscience and Bioengineer.*, 88(6), 627-631 (**1999**).
[23]. P. Sasi, R. R. Mehrotra, and M. Debnath, *Indian J. of Biotechnol*., 5, 364-367 (**2006**).
[24]. F. Hasan, A. A. Shah, A. Hameed, *Enzym. Microbiol. Technol*., 39, 235-251 (**2006**).
[25]. U.T. Bornscheuer, *FEMS Microbiol. Rev.*, 26, 73-81(**2002**).