ABSTRACT

The present study describes the isolation, identification and screening of fungal strain *Rhizopus oligosporus* var. *microsporus* (Saito) Schipper & Stalpers, (1984) for the production of extracellular lipases. One hundred and sixty seven cultures of fungi were isolated from different environments such as soil, air, milk, pickle, oily bread, destroyed fruits and vegetables by serial dilution method. The strains were initially selected qualitatively on Tween 80-Agar plates and were shifted to the slants of PDA (Potato Dextrose Agar) for maintenance and storage at $4^{o}C$. Quantitative screening for extracellular lipase production by isolated strains was carried out in shake flasks and the most potent strain, IIB-63 producing 3.20 ± 0.003 U mL^{-1} of enzyme was selected. The strain was then identified on the basis of standard morphological measurements and was assigned the code IIB-63.

The selected strain was then subjected to physical (UV and Gamma radiations) and chemical mutagenic (NA, EtBr, MNNG/NTG) treatments in order to improve its lipolytic potential. During the treatment mutants were qualitatively and quantitatively selected and IIB-63NTG-7 was found to be the mutant showing highest lipases production (10.37 ± 0.06^{a} U mL^{-1}) with a zone size of 12.3 mm on Luria-Bertani-tributyrin agar plates. This mutant showed an overall 325% increase in activity over its parent strain for the production of extracellular lipase.

The fermentation experiments for the production of extracellular lipases by wild and mutant strains were carried out in 250 mL Erlenmeyer flasks and laboratory scale 5L stirred fermenter. The cultural conditions were optimized for both wild and mutant strains of *R. oligosporus.*

Seven different culture media were tested for the production of extracellular lipase by both wild and mutant strain of *R. oligosporus* in shake flask fermentation. Of all the media evaluated M5 (gL^{-1} Peptone: 20, Glucose: 10, K_2HPO_4:2.0, $MgSO_4.7H_2O$: 0.12, NH_4Cl: 1.0, Yeast Extract: 2.5, pH: 7.0) gave highest units of extracellular lipases 3.16 ± 0.02^{a} U mL^{-1} (W) and 10.99 ± 0.02^{a} U mL^{-1} (M). Other culture media gave lesser production of enzyme by both wild (M2>M7>M3>M4>M1>M6) and mutant

(M1>M2>M3>M4>M7>M6) strains. The production of enzyme was found to be highly significant ($P \leq 0.05$) in media M5.

The effect of incubation temperature (15 -45°C), initial pH (4.0.-10.0), inoculum size (0.5-3.5 mL) and volume of the medium (25-150 mL) on the production of extracellular lipase by both wild and mutant strains was investigated in shake flask. The rate of fermentation was also studied and found maximum extracellular lipase was obtained after an incubation of 48 h by both wild and mutant strains.

Various agro industrial by-products (CSM: cotton seed meal, SBM: Soybean meal, WB: Wheat Bran, WF: Wheat Flour, SFM: Sunflower meal, AM: Almond meal, RB: Rice Bran) were tested for their effect on lipases production. In the presence of SBM (0.4%) the maximum lipolytic activity was 5.09 ± 0.008^{a} U mL^{-1} (W) & 16.43 ± 0.005^{a} U mL^{-1} (M) which was approximately 1.27 (W) & 1.42 (M) times higher than that in the absence of additive.

Different additional carbon sources was added to basal medium with the aim of improving extracellular lipases production. In the present study effect of different carbon sources such as lactose, maltose, sucrose, xylose, dextrose, glucose, starch and Tween 80 were evaluated for the production of extracellular lipases by wild and mutant strains of *Rhizopus oligosporus*. Of all the carbon sources tested, Tween 80 showed considerable increase in lipases production by both wild (5.52 ± 0.005^{a} U mL^{-1}) & mutant (19.13 ± 0.005^{a} U mL^{-1}) strains as compared to others.

Marked increase in the productivity of the enzyme has been observed upon addition of some nitrogen additives compared with the non-supplemented medium. Different organic nitrogen sources such as peptone, *p*-nitrophenol, casein, nutrient broth, urea, yeast extract and corn steep liquor were independently added to the fermentation medium. Maximum extracellular activity of lipase 5.85 ± 0.01^{a} U mL^{-1} (W) & 28.32 ± 0.01^{a} U mL^{-1} (M) was obtained when 0.8 % of casein was added in the fermentation medium as an organic nitrogen source. Different Inorgaic salts used are ammonium chloride [NH_4Cl], ammonium sulfate [$(NH_4)_2SO_4$], ammonium nitrate [NH_4NO_3], ammonium acetate [NH_4CH_3COO], ammonium ferro (II) sulfate 12- hydrate [$(NH_4)_2$ Fe $(SO_4)_2.12H_2O$], hydroxyl ammonium chloride [$HONH_3Cl$], Ammonium oxalate [$(NH_4)_2C_2O_4$] and ammonium molybdate [$(NH_4)_6MoO_{24}$]. Maximum extracellular production of lipases 8.31 ± 0.01^{a} U mL^{-1} (W) and 34.34 ± 0.01^{a} U mL^{-1} (M) were

observed when 0.8 % $(NH_4)_2C_2O_4$ was added in the substrate as an additional inorganic nitrogen source.

The rate of fermentation by extracellular lipases by both wild and mutant strains of *R. oligosporus* var. *microsporus* was investigated in stirred fermenter. It was found that the growth and lipases production was increased gradually and reached its maximum 9.07± 0.42[a] U mL^{-1} (W) & 42.49 ± 3.91[a] U mL^{-1} (M) after 30 h of fermentation for both wild and mutant strain. There is overall increase of 109% (W) and 124% (M) in the production of extracellular lipases as compared to shake flask. Another significant finding of the present experiment is that the fermentation period is reduced to 30 h in case of wild and 23 h in case of mutant from 48 h in shake flask studies.

Effect of different sizes of inoculum was investigated for extracellular lipases production by both wild & mutant strains of *R. oligosporus* var. *microsporus* IIB-63 in stirred fermenter. The size of the vegetative inoculum was varied from 1-5% and fermentation was carried out. It was observed that lipases activity of the both wild (13.76 ± 0.99[a] U mL^{-1}) and mutant (46.34 ± 3.05[a] U mL^{-1}) strains was gradually increased with the increase of inoculum size and reached its maximum at 3% of inoculum.

The initial pH of the fermentation medium was varied from 7.0 to 9.0 and was controlled throughout the fermentation process however the experiment with uncontrolled pH was also carried out. The production of extracellular lipases was found maximum 30.39 ± 2.58 U mL^{-1} (W) & 50.42 ± 4.37 U mL^{-1} (M) when the pH of the medium was maintained at 8.0. However in the experiment with uncontrolled pH there is no remarkable increase in the production of enzyme.

The rate of the agitation was varied from 150-300 rpm. Maximum enzyme production by both wild (27.30± 1.98 U mL^{-1}) and mutant (54.01± 4.54 U mL^{-1}) strains was obtained when the agitation speed was maintained at 250 rpm. Change in the rate of agitation resulted in decreased enzyme production. The rate of aeration was varied from 0.2-1.0 vvm. Production of enzyme by mutant strain was found maximum i.e., 59± 4.88 U mL^{-1} when the aeration rate was set at 0.8 vvm while wild strain gave maximum lipase units (28± 1.97 U mL^{-1}) at 1.0 vvm.

The enzyme produced after optimization of the cultural conditions was subjected to ammonium sulfate precipitation for salting out the proteins. 60% ammonium sulfate showed the enzyme activity of 11.45 U mL^{-1} by wild and 28.2 U mL^{-1} by mutant strain of *R. oligosporus* var. *microsporus*. While in 80% ammonium sulfate the enzyme activity by

both the wild (13.14 U mL^{-1}) and mutant (29.5 U mL^{-1}) strains increased which indicates the partial purification of enzyme. Desalted enzyme was subjected to DEAE-cellulose column for ion exchange chromatography. Wild strain shows 206.73 fold purification and 82.56% recovery while the mutant strain shows 407.34 fold purification with 62.83% recovery. Sephadex G-100, is a cross-linked polymer used for gel filtration chromatography, which is used for differentiating the molecular size. There is 446.19 fold (W) and 710.02 fold (M) purification of enzyme which shows that most of the contamination proteins are removed.

The effect of pH, temperature and metal ions was also investigated on the activity of purified lipases. It is evident from the results that the maximum residual activity by both strains 81% (W) and 100% (M) was observed in the reaction mixture of pH 8.0. The results showed that lipases retained 80% of its activity at $25^{o}C$-$30^{o}C$ by wild and 100% of its activity at $20^{o}C$-$50^{o}C$ by mutant strain of *R. oligosporus* var. *microsporus.* Mn^{++} stimulated the activity of lipases by the wild while it has inhibitory effect on lipases activity of mutant strain. Other ions Like Ca^{++}, K^{+}, Mg^{++}, Cu^{++} and Na^{+} stimulated the activity of lipases by both wild and mutant strains. Both wild and mutant strains showed the same response of inhibition of enzyme activity in the presence of Hg^{++} and Fe^{++}.