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Production and optimization of lipase using *Aspergillus niger* MTCC 872 by solid-state fermentation

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Abstract

Background: Lipases are serine hydrolases that degrade triglycerides, an attribute that treasures wide applications in biodiesel production, detergent, chemical industries, etc. The most sought after the application is in the high quality and economical production of biodiesel under mild reaction conditions and simplified product separation. For the said application, fungal lipases are ideal catalysts that could effectively catalyze esterification and transesterification reactions with their specific ability to release fatty acids from 1, 3 positions of acylglycerols.

Results: In the present work, to facilitate bulk synthesis, lipase production using *Aspergillus niger* MTCC 872 was studied by solid-state fermentation (SSF). The chosen fungal strain was evaluated for lipase production using a mixture of agro-industrial substrates viz. rice husk, cottonseed cake, and red gram husk in various combinations at flask level. Tri-substrate mixture (rice husk, cottonseed cake, and red gram husk) combined in the ratio of 2:1:1 has shown the maximum lipase activity 28.19 U/gds at optimum cultivation conditions of temperature 40 °C, moisture content 75% (*v/w*), pH 6.0 and initial spore concentration of 5.4 million spores per mL. Further studies were performed for scale-up of lipase from flask level to lab scale using tray fermenter. Lipase activity was found to be 24.38 U/gds and 21.62 U/gds for 100 g and 1000 g substrate respectively.

Conclusion: This is the first report on the production of lipase from *Aspergillus niger* MTCC 872 using tri-substrate mixture of rice husk (RH), cottonseed cake (CSC), and red gram husk (RGH). Moreover, comparison between individual, binary, and tri-substrate mixture was carried out for which the highest lipase activity was observed for tri-substrate mixture. In addition, comparable results were found when scale-up was performed using tray fermenter. Thus, the current work signifies usage of agro-industrial residues as substrates for enzyme production by solid-state fermentation process as an effective alternative to submerged fermentation for industrial applications.

Keywords: Lipase, Solid-state fermentation (SSF), Agro-residues, Solid tri-substrate

Introduction

Over the recent years, lipases have emerged as one of the prominent biocatalysts and accounts nearly 10% of the enzyme market (Salihu et al. 2016). Fossil fuels are running out of reserves due to enormous usage in vehicular applications. Therefore, there is an imperative need to produce eco-friendly and clean energy alternatives like biodiesel in an economical and environmentally sustainable method. For this, lipases are extremely important as they hydrolyze acylglycerol into fatty acid and glycerol, and effect esterification and transesterification reactions (Stamenković et al. 2011; Christopher et al. 2014; Selvakumar and Sivashanmugam 2017). In general, lipases are triacylglycerol acyl hydrolases (EC 3.1.1.3), classified as serine hydrolases that catalyze triglycerides into diglycerides, monoglycerides, and fatty acids (Kanmani et al. 2015).

Lipases have proven potential for contributing to the multibillion-dollar market share in bio-industry and are being used in the processing of medicines (digestive enzymes), detergents (cleaning agent), food additives (flavor modifying enzymes), paper (control of pitch), cosmetics (exclusion of lipids), leather (elimination of fat

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from animal skin), wastewater (decomposition and oil removal), biodiesel production (transesterification reaction), etc. (Aravindan et al. 2007; Joseph et al. 2008; Andualema and Gessesse 2012; Selvakumar and Sivashanmugam 2017). Lipases are also used in the degradation of fatty wastes and biodegradation of polymers (Gombert et al. 1999; Sharma et al. 2001; Kanmani et al. 2015). Due to their distinctive huge catalytic potential, lipase is considered as one of the most crucial industrial enzymes (Saxena et al. 1999; Andualema and Gessesse 2012).

Lipases occur widely in plants, animals, and various microorganisms (Gilham and Lehner 2005; Show et al. 2015). In comparison to enzymes derived from animals and plants, enzymes originated from microbes are the better sources as they provide high stability and offer ease of cultivation. Microbial lipases which are used for commercial applications act as catalysts for a wide range of hydrolytic and synthetic reactions (Andualema and Gessesse 2012). Kynclova et al. (1995) screened various hydrolytic enzymes to meet the special demands. Among the screened microorganisms, fungal lipases turned out to be the best source for lipase as they are thermally stable with high turnover number and are presently receiving attention due to the easy recovery of extracellular enzymes (Kynclova et al. 1995; Mahadik et al. 2002; Salihu et al. 2013; Fleuri et al. 2014).

Industrially, lipases have been produced using submerged fermentation (SmF). Problems associated with this process include high cost of equipment, media, and higher probability of contamination. The traditional and emerging field of solid-state fermentation (SSF) has enabled the better production of a wide range of enzymes and metabolites which require less energy than SmF (Pandey 2003; Viniegra-González et al. 2003; Ashok et al. 2017). The credibility of solid-state fermentation (SSF) for production of enzyme has gained importance in developing countries (Singhania et al. 2009). SSF technology has been refined over the years and is currently the best method of obtaining fungal spores on the non-soluble matrix that acts as a natural habitat for filamentous fungi (Hölker et al. 2004; Colla et al. 2015). SSF also offers other advantages including resistance to contamination and ease of product extraction, and provides a better opportunity for the biosynthesis of low-volume-high-cost products. This is achieved by using low cost accessible agro-industrial residues as solid substrates, which provide both anchorage and nutrient source to microbial cells (Krishna 2005; Ashok et al. 2017). These agricultural residues are being generated in huge quantities in developing countries and problems associated with their disposal is also an important environmental concern. Thus, utilizing these residues as nutrient source for enzyme production will reduce overall production cost (Salihu et al. 2013). Hence, the selection of an appropriate substrate is an essential step during the production of enzymes in SSF. Various agro-industrial residues such as wheat bran, soybean cake, rice husk, gingelly oil cake, olive oil cake, sugar cane bagasse, babassu oil cake, and sheanut cake were studied (Salihu et al. 2013, 2016; Fleuri et al. 2014).

In the present study, we first explored the possibility of using a mixture of agro-industrial residues for the better production of Lipase using *Aspergillus niger* MTCC 872. In the next stage, physical parameters such as temperature, pH, moisture content, and initial spore concentration were optimized using one variable at a time method. The procedure adopted here for evaluating physical parameters was to evaluate the effect of individual factor and incorporate optimized level in the next factor optimization. In addition, optimized results were transferred to tray bioreactor at the laboratory scale and the efficacy of the strain for lipase production was evaluated.

Materials and methods

Microorganism

Aspergillus niger MTCC 872 was obtained from Microbial Type Culture Collection Centre and Gene Bank, Institute of Microbial Technology, Chandigarh, India. Obtained fungal strain was revived and grown on potato dextrose slant at 30 °C for 96 h and stored at 4 °C. Fungal spores were harvested with distilled water and obtained spore concentration was determined using Neubauer chamber.

Solid substrates and chemicals

Rice husk (RH), cottonseed cake (CSC), and red gram husks (RGH) were procured from the local market in Hyderabad, India. Other media components used in the experiment were obtained from Hi-media (Mumbai, India). All the chemicals were of analytical reagent grade.

Solid-state fermentation and extraction of the enzyme

Rice husk, cottonseed cake, and red gram husk were tested in various combinations (individual, combination of two and mixture of all the three substrates) by fixing the total weight to 5 g for lipase production. Five grams of substrate mixture was taken in a 250-mL Erlenmeyer flask and supplemented with 0.05 M potassium phosphate buffer (K_2HPO_4 and KH_2PO_4) to maintain an initial moisture content and pH. The flasks were sterilized by autoclaving at 121 °C (15 psi) for 15 min. After cooling, 4% (*v*/*w*) inoculum of the fungal strain *Aspergillus niger* MTCC 872 was added to the solid substrate. The flask was incubated at a temperature of 30 °C for 96 h (Edwinoliver et al. 2010; Dayanandan et al. 2012).

Solid substrate with fungal biomass was withdrawn at regular intervals under sterile conditions. In order to get a fully representative sample, the fermented mixture was

mixed thoroughly to get the uniformity. 0.5 g of moldy substrate was mixed with 10 mL of solution containing 1% (*w*/*v*) NaCl and 1% Triton X-100 (*w*/*v*). This mixture was ground using mortar and pestle. The resulting solution was then filtered using Whatman No. 1 filter paper. The residue left in the filter paper was dried at 70 °C for 24 h to obtain the dry solid weight. The filtrate was then centrifuged at 10,000 rpm for 10 min. The resultant supernatant was used as an enzyme source for Lipase assay represented in Fig. 1. All the analysis was done in triplicates. In order to evaluate optimized parameters, the effect of individual parameters was evaluated.

Scale-up to 1 kg capacity

The flask level studies were scaled up to 100 g of an optimized solid mixture containing rice husk, cottonseed cake, and red gram husk and were transferred to a 1-L Erlenmeyer flask, which provided sufficient surface area for the growth of microorganism on the solid substrates. The mixture was moistened with buffer and sterilized at 121 °C for 15 min. After cooling the substrates to room temperature, the inoculum was added and incubated for 96 h. Likewise, further scale-up in a tray bioreactor was performed using a steel tray of dimensions: length, 45.2 cm; breadth, 42.7 cm; and height, 2.5 cm. One kilogram of solid tri-substrate mixture was moistened (75% *v*/*w*) with buffer of pH6.0 and sterilized. Solid mixture was distributed on trays and once cooled, the inoculum was added and mixed inside laminar airflow chamber and kept for fermentation at 40 °C. Sampling was performed in tray bioreactor after complete manual mixing of all the substrate-biomass mixture. Samples were collected at every 24 h interval and analyzed for lipase activity (Doriya and Devarai 2018).

Lipase assay

Lipase activity was determined using the modified titrimetric method (Arzoglou et al. 1994). The reaction mixture containing 5 mL of olive oil emulsion substrate, 20 mL of 0.1 M potassium phosphate buffer, and 1 mL of enzyme extract was mixed and incubated in a rotary shaker at a temperature of 30 °C at 130 rpm for 30 min. This reaction mixture was then quenched using 15 mL acetone-ethanol (1,1) mixture. The amount of fatty acids released was titrated against 0.05 N NaOH until the solution turned into pink color. Blank assays were conducted adding the enzyme just before titration. One unit (U) of lipase activity is defined as the amount of enzyme which produces 1 μmol of fatty acids per minute under the assay conditions. In general, enzyme activity can be expressed in terms of units per milliliter or units per gram dry substrate.

$$\text{Lipase activity } (U/L) = \frac{A * C}{V} * 10^6$$

Where A = mL of NaOH consumed per minute; U = μmoles of fatty acids released per minute.

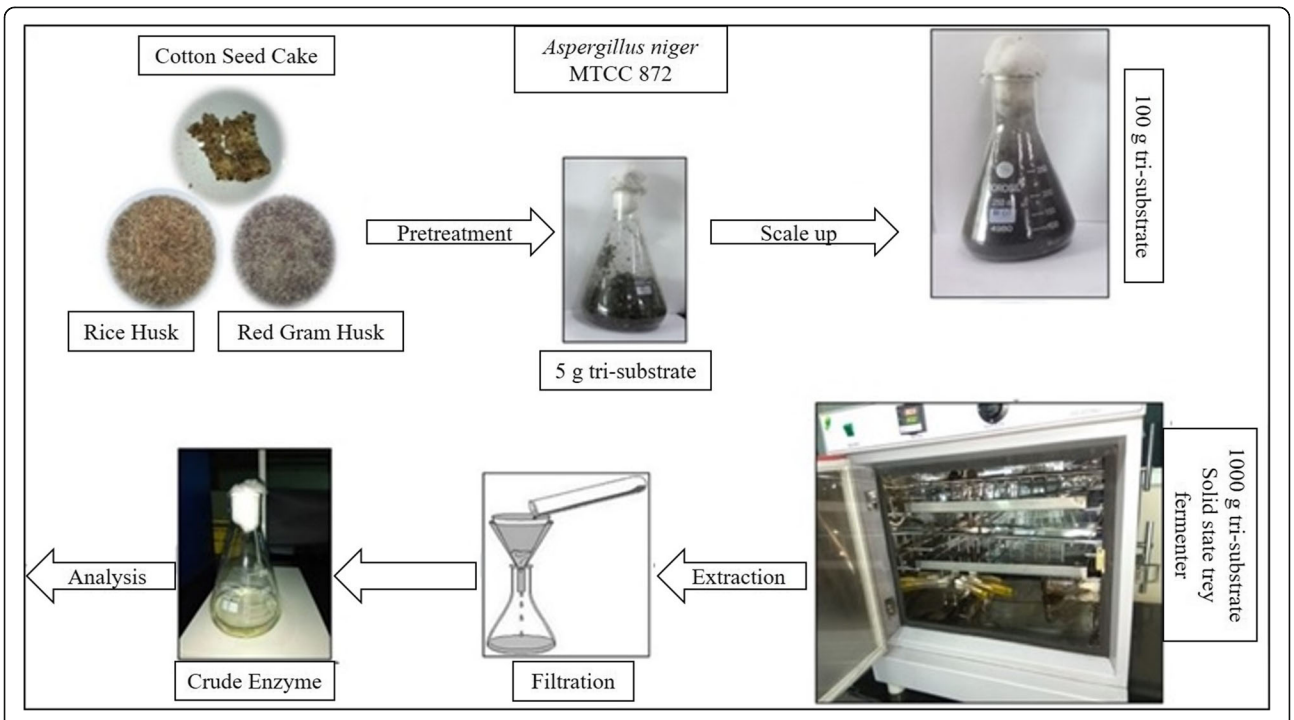

Fig. 1 Graphical abstract of lipase production using solid-state fermentation with *Aspergillus niger* MTCC 872

C = concentration of NaOH in mmoles/L; V = enzyme sample volume in μL; 10^6 converts sample volume to liters.

U/mL = μmoles of fatty acids released per minute per mL of crude enzyme; U/gds = μmoles of fatty acids released per minute per gram of dry solid substrate from which enzyme has been extracted.

Results

Effect of solid substrate

In the current work, the effect of three different agricultural residues, rice husk, cottonseed cake, and red gram husk, were evaluated using solid-state fermentation. Effect of individual solid substrate on lipase activity is represented in Fig. 2a. Highest lipase activity of 7.17 U/gds was observed for RGH after 24 h, and the lowest activity of 4.2 U/gds was observed using RH. Effect of binary mixture with equal ratio is shown in Fig. 2b. Maximum lipase activity for RGH:RH binary mixture was observed as 9.89 U/gds after 72 h of fermentation. SSF process was also studied using ternary substrate mixture (RH:CSC:RGH) by varying proportions of the components. SSF was carried out at 30 °C with an initial moisture content of 60% for 72 h. As represented in Fig. 2c, lipase activity ranged from 6.26 U/gds to 12.93 U/gds using ternary mixture in different ratios for the production of lipase using *Aspergillus niger* MTCC 872. Maximum lipase activity of 12.93 U/gds was obtained for the substrate ratio 2:1:1 (RH:CSC:RGH) and minimum lipase activity of 6.26 U/gds for the substrate ratio 1:1:2 after 24 h of fermentation.

Effect of temperature

Effect of temperature was studied using fermentation with optimized substrate concentration by incubating at different temperatures ranging from 30 °C to 45 °C. Optimal lipase activity of 13.07 U/gds was found using mixed substrate 2:1:1 at 40 °C at 48 h as shown in Fig. 3a.

Effect of pH

SSF was carried out using previously optimized tri-substrate mixture with composition 2:1:1 (RH:CSC:RGH) at 40 °C and moisture content at this stage was maintained at 60%. During the pre-treatment, pH of tri-substrate mixture is varied from 5.5 to 8 with an increment of 0.5. Influence of pH was investigated by measuring lipase activity as shown in Fig. 3b. Maximum lipase activity of 25.12 U/gds was observed at pH 6. On further increasing in the pH, decline in lipase activity, i.e., at pH 7.5 lipase activity of 7.14 U/gds was observed.

Effect of moisture content

To evaluate the impact of moisture content on lipase production, experiments were conducted using the aforementioned optimum conditions for tri-substrate

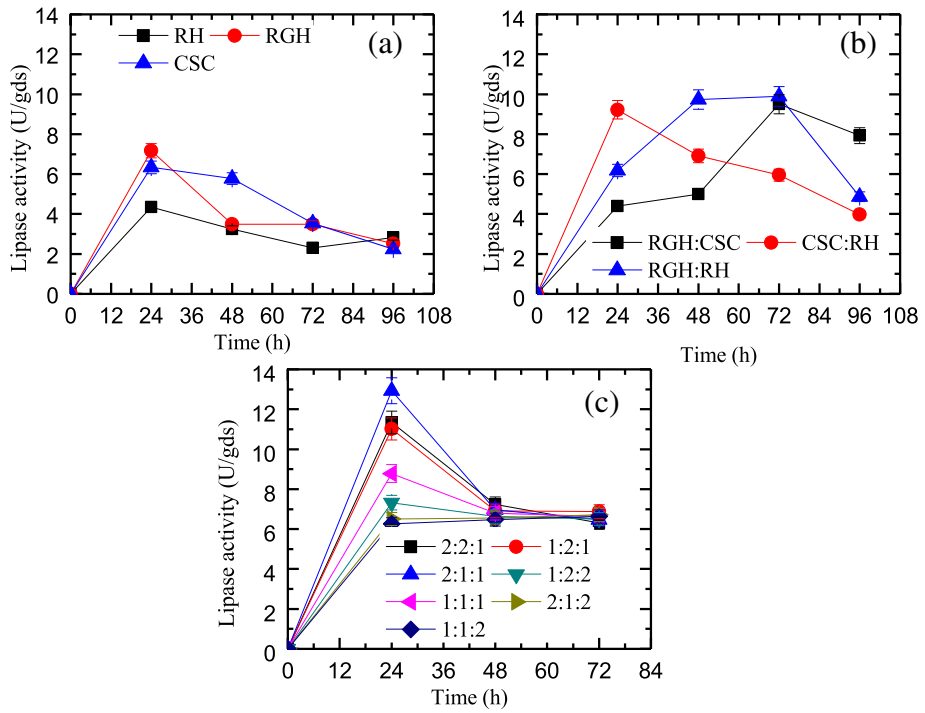

Fig. 2 Lipase activity (U/gds) with different solid substrates (RH, RGH, and CSC) compositions. **a** Individual substrates. **b** Mixture of two substrates. **c** Mixture of three solid substrates

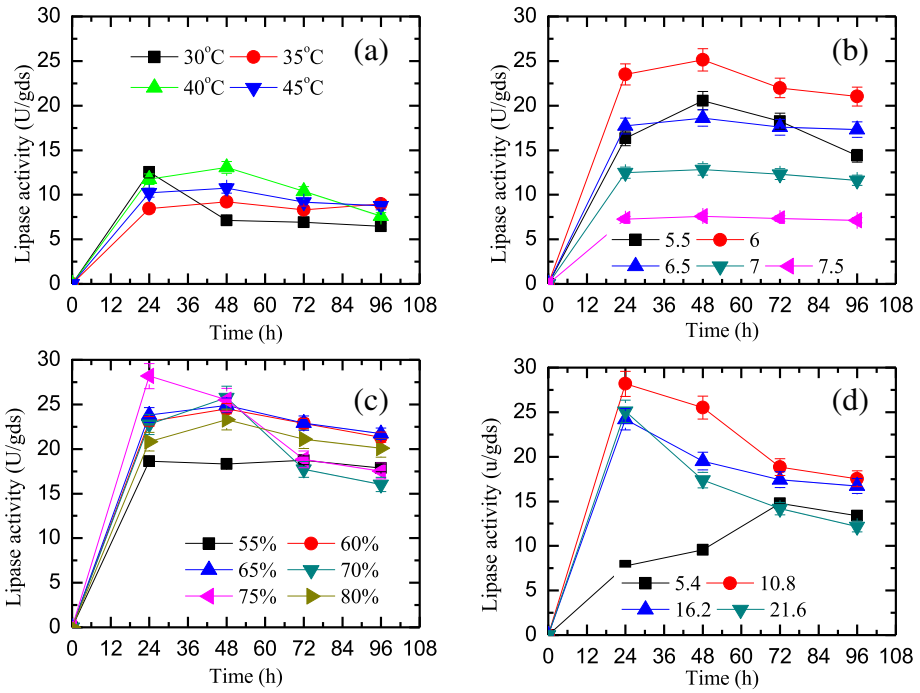

Fig. 3 Evaluation of optimum conditions for maximum lipase activity using *Aspergillus niger* MTCC 872. **a** Temperature. **b** pH. **c** Moisture content (% *v/w*) **d** Initial cell concentration (million spores /mL)

mixture. Lipase activity was obtained by varying moisture content from 55% to 80% (*v*/*w*) using phosphate buffer solution (pH 6) prior to fermentation as represented in Fig. 3c. Maximum lipase activity of 28.19 U/gds was observed at 75% moisture content after 24 h fermentation. Increase in lipase activity was observed with increase in moisture content from 55 to 75% (*v*/*w*). On further increment, decrease in lipase activity was observed.

Effect of spore concentration

To study the effect of initial cell concentration, experiments were conducted with aforementioned optimized parameters. Lipase activity was determined for solid tri-substrate inoculated with 4% (*v*/*w*) of *Aspergillus niger* MTCC 872 suspension consisting of 5.4, 10.8, 16.2, and 21.6 million spores per mL and shown in Fig. 3d. Highest lipase activity was 28.19 U/gds observed at cell concentration of 10.8 million spores per mL after 24 h.

Scale-up using tray bioreactor

Experiments were carried out in a tray bioreactor under the condition in which 100 g and 1000 g of optimized substrate mixture was fermented using previously optimized cultivation conditions. After 48 h of fermentation, the lipase activity was found to be 24.38 U/gds and 21.62 U/gds for 100 g and 1000 g substrate respectively as shown in Fig. 4. Lipase activity using different solid substrate at optimized conditions from *Aspergillus* species are shown in Table 1.

Discussion

SSF involves the growth and metabolism of the microorganisms on the moist solid substrate without any free-flowing water. Three different agricultural by-products rice husk, cottonseed cake, and red gram husk were evaluated for lipase activity with individual, binary mixture in equal

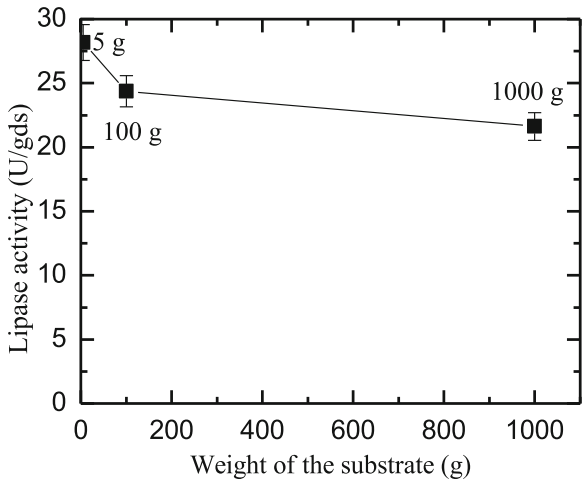

Fig. 4 Scale-up of lipase production in flask and tray bioreactor at optimum conditions

Table 1 Comparison of various works on lipase production

S. no.	Microorganism	Substrate	Optimum conditions	Lipase activity (U/gds)	Reference
1.	*Aspergillus niger* (O-4)	Wheat bran, rice husk	35 °C, pH– 6	42.82 ± 1.65	(Colla et al. 2015)
2.	*Aspergillus niger*	Shea butter cake	30 °C, 168 h, pH– 7.0	3.35	(Salihu et al. 2013)
3.	*Aspergillus niger*	Rice bran	30.3 °C, pH– 6.87	121.53	(Hosseinpour et al. 2012)
4.	*Aspergillus niger* MTCC 2594	Wheat bran, Coconut oil cake and Wheat rawa	30 °C, 72 h, pH– 7.0, MC 60%	745.7 ± 11	(Edwinoliver et al. 2010)
5.	*Aspergillus niger* MTCC 2594	Gingelly oil cake	30 °C,120 h, pH– 7.0, MC 60%	58.6	(Damaso et al. 2008)
6.	*Aspergillus niger* J-1	Olive oil and Glucose	30 °C, 168 h, pH– 7.0	9.14	(Falony et al. 2006)
7.	*Aspergillus niger* NCIM 1207	Wheat bran	45 °C, pH– 2.5	630	(Mahadik et al. 2002)
8.	*Aspergillus niger* MTCC 872	Rice husk, cotton seed cake and red gram husk	40 °C, pH– 6, MC 70%	27.2 (5 g) 24.38 (100 g) 21.62 (1000 g)	Current work

ratios and tri-substrate mixture in different ratios represented in Fig. 2a, b, and c, respectively. Nutrient sources (primarily carbon and nitrogen) and surface area are the two major factors that support fungal growth on the solid substrate. Highest lipase activity was observed for RGH and nearly similar activity for CSC after 24 h, whereas RH has shown lowest activity. Figure 2b represents binary mixture of substrates with equal ratio and maximum lipase activity was observed for RGH:RH after 72 h of fermentation. At the same time, comparable activity was observed for the mixture of RGH and CSC. Mixture of RGH:RH shows growth in the activity till 48 h and then retaining its activity till 72 h with a small increment. In case of RGH:CSC, rapid growth in activity was observed from 48 h to 72 h. Mixture of CSC:RH has shown notable growth for 24 h, thereafter gradual decline in the activity was observed. Binary mixtures taken in equal ratios have shown higher activity when compared with individual substrate. Similar results were observed when SSF was conducted with different combinations of rice bran, sugar cane bagasse, and wheat bran. Maximum lipase activity with mixed substrate of sugarcane bagasse and wheat bran was observed whereas for individual substrate the activity was found to be lower (Babu and Rao 2007). In the next stage of present work, SSF process was studied using ternary substrate mixture in the ratio of RH:CSC:RGH. Fermentation was carried out at 30 °C with an initial moisture content of 60% for 72 h. Figure 2c shows lipase activity for ternary mixture taken in different ratios using *Aspergillus niger* MTCC 872, representing the significance of selected solid substrates and their mixture composition. Maximum lipase activity was obtained for the substrate ratio 2:1:1 (RH:CSC:RGH) after 24 h of fermentation. With 2:2:1 and 1:2:1 tri-substrate mixtures, lipase activity was found to be similar. Lowest lipase activity was observed with tri-substrate composition of 1:1:2. Of largely, it was observed that the variation in the ratio of substrate mixture would influence the lipase activity. The above

results suggest that RGH and CSC are the primary nutrient source whereas RH plays a key role in providing sufficient surface area to promote fungal growth. To date, several studies were conducted to optimize fermentation conditions for lipase production using individual substrate, but very few have evaluated effect of different combinations of substrate during.

In the present work, ternary mixture has given maximum activity when compared to binary and individual substrate. This observation was in line with study conducted by Edwinoliver et al. (2010), wherein mixture of different substrates improved the growth and enzyme production, as it is difficult to acquire all essential nutrients from single substrate (Edwinoliver et al. 2010). In another study, high glutaminase activity was observed using a mixed substrate than single substrate (Sathish et al. 2008). Further physical parameters were optimized for this mixture composition and translated to a tray bioreactor for scale-up studies. Figure 3a represents the effect of temperature on the lipase activity. Optimized substrate concentration was incubated at different temperatures ranging from 30 °C to 45 °C with an increment of 5 °C. At 30 °C, lipase activity increased for 24 h thereafter sudden drop in the activity was observed. Enzyme activity increased with increase in temperature till 40 °C. Further increase in temperature led to decrease in biosynthetic activity. Similar results were observed where *Aspergillus niger* strain was optimally active in the temperature range of 40 °C to 60 °C during lipase production (Falony et al. 2006). Optimal lipase activity was found using mixed substrate 2:1:1 at 40 °C and 48 h as shown in Fig. 3a. This is in line with the study conducted by Kamini et al. (1998) where *Aspergillus niger* strains have been reported to be active between 40 °C

and 55 °C. Effect of pH is essential during SSF as change in pH could impact microbial growth and lipase activity. The influence of pH was investigated by varying the pH of potassium phosphate buffer ranging from 5.5 to 8. The pH affects stability of enzymes by changing the electrostatic interactions of their protein structure, causing changes in the amino acids ionization status, which defines the secondary and tertiary structures of protein and therefore its activity and stability (Eerappa et al. 2008). Optimum pH was determined by measuring lipase activity under fermentation conditions of 40 °C and moisture content 60% using optimized substrate mixture represented in Fig. 3b. Initially, lipase activity increased with increase in the pH and maximum lipase activity was observed at pH 6. On further increasing the pH, decrement in the lipase activity was observed. Another study reported that *Aspergillus niger* possesses good pH stability (pH 4–0–10.0) during lipase production (Kamini et al. 1998). Higher pH ranging from 7.5 to 8.0 did not show significant effect on lipase production by *Aspergillus niger* MTCC 872. Optimum moisture content and water activity are extremely important for growth of microbes during solid-state fermentation. Numerous fungi grow in low-moisture environment and hence they are quite adaptable to SSF technique when compared to bacteria. In the current work, lipase production was affected by moisture content variation as shown in Fig. 3c. Maximum lipase activity was observed at 75% moisture content after 24 h fermentation. Most of the reports suggest that maximum lipase activity is obtained at moisture content ranging from 60 to 80%. Also, higher moisture content would lower the substrate porosity thereby affecting fungal growth. At moisture level of 55 to 75%, lipase activity increased. Low moisture level would result in minimal growth due to a reduction in nutrient diffusion and low substrate swelling (Baysal et al. 2003). In contrast, 50% of moisture was optimal for protease and lipase production from *Pseudomonas aeruginosa* using Jatropha seed cake as a substrate (Mahanta et al. 2008). In another study, maximum lipase production from *Aspergillus niger* was observed at 71% moisture content using wheat bran as substrate (Mahadik et al. 2002). Hence, initial moisture content during the SSF technique would influence the substrate medium thereby affecting the microbial growth and substrate decomposition. Maximum lipase activity was obtained with optimized parameters for substrate ratio, temperature, pH, and moisture content of 2:1:1 (RH:CSC:RGH), 40 °C, pH 6, and 75% respectively. Further experiments were conducted to study the effect of initial cell concentration shown in Fig. 3d. At lower cell concentration of 5.4 million spores per mL, slow growth was observed since most of the nutrients were utilized for increasing cell concentration. Highest lipase activity was observed at cell concentration of 10.8 million spores per mL after 24 h. On further increasing the initial cell concentration decrease in lipase activity was observed, this might be due to rapid depletion of nutrient sources in the media to sustain the overall cell concentration. Scale-up of lipase production using 100 g and 1000 g substrate mixture resulted in 89.33% and 79.48% of the enzyme activity at flask level. Similar results were obtained, when scale-up was carried out for mixture of wheat bran and gingelly oil cake for which lipase activity of 95% and 84% of flask level for 100 g and 1000 g respectively was observed (Mala et al. 2007). In another study using wheat bran, coconut oil cake, and wheat rawa, lipase activity for 100 g and 3 × 1000 g was found to be 96% and 83% of the flask level lipase activity using *Aspergillus niger* MTCC 2594 (Edwinoliver et al. 2010).

Conclusion

Based on the current study, it can be concluded that *Aspergillus niger* MTCC 872 is an acidic lipase producer using substrate mixture composed of rice husk, cotton seed cake, and red gram husk which is easily available across the Indian markets. From the results, it can be perceived that CSC and RGH are the key nutrient component and RH provides sufficient surface area for fungal growth. The optimum conditions for substrate ratio, temperature, moisture content, and pH are found to be 2:1:1, 40 °C, 75% and 6.0 respectively. They also play a crucial role in lipase production. Lipase activity in tray bioreactor was found to be slightly lower, in comparison to SSF conducted in flasks. In conclusion, *Aspergillus niger* MTCC 872 strain and the solid substrates used in the present work are promising sources of lipase production.

Abbreviations

CSC: Cotton seed cake; RGH: Red gram husk; RH: Rice husk; SSF: Solid-state fermentation

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Availability of data and materials

All the data generated and analyzed during the study are included in the article.

Authors' contributions

AN, VM, and SHP performed the experiments, and SK supported during the manuscript preparation. SKD guided AN, VM, and SHP during the experimentation and manuscript preparation. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors have read and approved to submit it to Bulletin of the National Research Centre. There is no conflict of interest of any author in relation to the submission.

Competing interests
The authors declare that they have no competing interests.

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