

The extraction of crude enzyme of lipase from *Penicillium candidum* PCA 1/TT031 by way of solid state fermentation

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Abstract

Solid-state fermentation (SSF), which is an excellent alternative for industrial enzyme production, entails the exploitation of cheap agro-industrial residues (low priced culture media). This paper delves into the cultivation of mould (GRAS *Penicillium candidum* PCA 1/TT031) using wheat bran (WB) as the culture media. The parameters for crude enzymatic extraction of lipase production were optimized to achieve the highest possible lipase activity. An incubation period of 7 days (3.06 U/g WB), 2% of tributyrin (5.43 U/g WB), meat peptone equal to 2% (5.2 U/g WB), moisture content of 50% (v:w) (6.6 U/g WB), initial pH of 9.0 (8.6 U/g WB), inoculum size of 5×10^6 spore/g of WB (11.3 U/g WB), an incubation temperature of 20°C (13.6 U/g WB), and an extractant consisting of phosphate buffer pH 7.0 (14.7 U/g WB) were the conditions applied for this study.

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Introduction

Solid-state fermentation (SSF), which is deemed eco-friendly, commonly uses solid agro-industrial wastes (residues) as substrates (source of carbon) (Thomas *et al.*, 2013). The SSF process has been extensively researched for the generation of high value-added products. These high value-added products include enzymes and biofuels (Singhania *et al.*, 2009). The employment of SSF for the production of enzymes (such as lipases and proteases) is favourable as it comes with several advantages (Salihu *et al.*, 2012). Amongst all the agro-industrial residues, wheat bran is considered the most appropriate for many secondary metabolisms. These include lipases and proteases (Couto and Sanromán, 2006; Barrios-González, 2012; Thomas *et al.*, 2013). A successful SSF process is dependent upon several significant factors. Among them are the selection of a suitable substrate and microorganism, product isolation, process optimisation, as well as purification (Thomas *et al.*, 2013; Murthy and Kusumoto, 2015). Lipases, which are considered

highly stable enzymes, can remain active even under extreme conditions. These enzymes can be obtained in satisfactory amounts from plants, animals and recombinant or natural microorganisms. Lipase production was compared by Diaz *et al.* (2006) for *Rhizopus homothallicus* in SSF cell cultures and submerged fermentation (SmF). A maximum extracellular lipase activity of almost 50 U/mL can be realized after a 22 hour fermentation period. The SSF cell cultures displayed maximum lipase activity of 1,500 U/g for dry matter (DM) following a 12 hour period of culture growth. The production of acidic lipase was studied by Mahadik *et al.* (2002). They employed *Aspergillus niger* in SSF through the optimisation of various factors on a 'one by one' basis. It was observed that the highest recovery was achieved through a combination of wheat bran and olive oil as the lipid substrate. In the context of enzyme production, wheat bran proved to be the most appropriate among other natural substrates.

Numerous factors govern SSF. Each factor is critical for establishing the economic and technical feasibility of the development process. While many

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of these factors are generic in nature, they need to be considered in a holistic manner as they still account for a significant impact (Thomas *et al.*, 2013). This study employs *P. candidum* PCA 1/TT031 during the SSF process to determine the optimum extraction parameters for the production of crude lipase.

Materials and methods

Inoculum preparation

Inoculum of lyophilized spores *P. candidum* PCA 1/TT031 [commercially freeze-dried strains from Chr. Hansen (Arpajon, France)] were prepared according to the procedure introduced by Murthy and Kusumoto (2015), albeit with minor modifications by replacement 1% Tween-80 in 0.01% Triton x-100.

Lipase production through *P. candidum* PCA 1/TT031 using wheat bran as the solid media

In order to reduce the cost of enzyme production, the conditions for microorganism cultivation need to be optimised (Maciel *et al.*, 2008). To determine the optimum values, different cultivation conditions were applied and studied. *P. candidum* PCA 1/TT031 was employed to realize the greatest amount of lipase. The SSF technique was harnessed for the one-factor-at-a-time (OFAT) approach to examine various parameters in crude lipase production. In subsequent experiments, parameters that were previously optimised were incorporated. Figure 1 shows the various parameters for obtaining the highest activity of crude lipase from *P. candidum* PCA 1/TT031 with wheat bran as the solid media.

The effect of time course on lipase production

A mass of 10 g wheat bran (with a particle size of 0.4 mm based on preliminary work) was transferred into 250 mL Erlenmeyer flasks and moistened with 65% of distilled water (Falony *et al.*, 2006). Subsequent to sterilization, the flasks were inoculated with 1×10^6 spore/g WB of *P. candidum* PCA 1/TT031 (Kotogán *et al.*, 2014) and incubated at a temperature of 25°C. The fermentation period stretched from 3 to 9 days (1 day interval). For the preparation of crude enzyme, the fermented culture was harvested with 50 mL of sterilized distilled water. This is in accordance to the procedure carried out by Malilas *et al.* (2013), albeit with minor modifications. Subsequently, a rotary shaker was employed (180 rpm for 30 minutes) to agitate the mouldy bran at room temperature. In order to segregate the tiny elements of the substrate, the broth extracts were accumulated, filtered through a muslin cloth and centrifuged (refrigerated centrifuge SIGMA 3–18 K Goettingen, Germany) at $5000 \times g$ for 20 minutes at a temperature of 4°C (Mahadik *et al.*, 2002). The resulting supernatants were assayed for lipase activity (Macedo *et al.*, 1997) and expressed as U/g WB.

The effect of nutritional factors on lipase production

The effect of different carbon sources on lipase production

The effects of carbon source 1% (w/w) on lipase production by *P. candidum* PCA 1/TT031 was assessed through the addition of various carbon sources to the wheat bran medium. The carbon sources added were

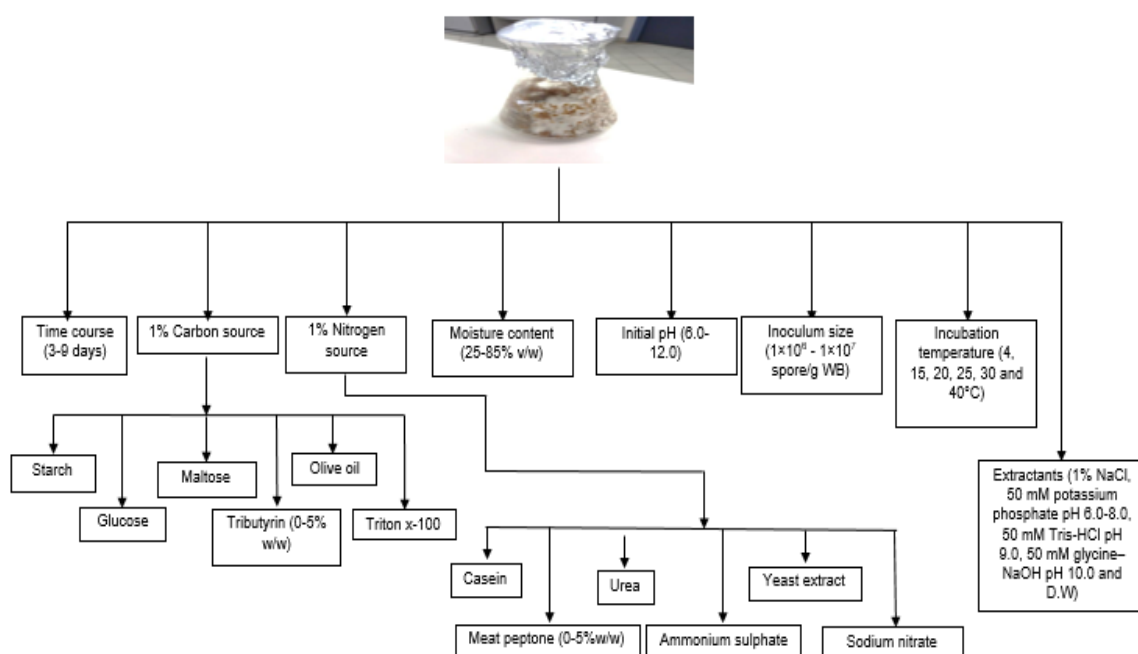


Figure 1: Optimization factors effecting on lipase production by *P. candidum* PCA 1/TT031 under solid-state fermentation

starch, tributyrin, olive oil, triton x-100, glucose and maltose. The best carbon source (1% tributyrin) for lipase production was selected to study the effect of different concentrations of tributyrin. A range of 0% to 5% (w/w) [Interval 1%] was added to 10 g of wheat bran.

The effect of different nitrogen sources on lipase production

Various nitrogen sources were tested. These included 1% (w/w) of casein, meat peptone, yeast extract, urea, ammonium sulphate ($(\text{NH}_4)_2 \text{SO}_4$), and sodium nitrate (NaNO_3). The nitrogen sources were added to 10 g of solid medium, 2% of tributyrin (optimum carbon source for lipase production).

The effect of physical factors on lipase production

The effect of different moisture contents on lipase production

The investigation on the effect of moisture content on lipase production involved the cultivation of *P. candidum* PCA 1/TT031 with different moisture concentrations of medium 25-85% (v/w) (interval 5%). Ten grams of wheat bran was supplemented with 2% of tributyrin and 2% of meat peptone (optimum nitrogen source for lipase production).

The effect of different initial pH levels on lipase production

The effect of pH on lipase production was explored by adjusting the pH level of the solid media from 4.0 to 12.0 (interval 1). Ten grams of wheat bran was supplemented with 2% of tributyrin, 2% of meat peptone, a moisture content of 50% (optimum moisture content for lipase production) and an inoculum size of 1×10^6 spore/g WB of *P. candidum* PCA 1/TT031. The pH levels were adjusted with the use of 1M NaOH and 1M HCl.

The effect of different inoculum sizes on lipase production

The generation of extracellular lipase by *P. candidum* PCA 1/TT031 at different inoculum sizes (1×10^6 - 1×10^7 spore /g WB) was studied.

The effect of different incubation temperatures on lipase production

In order to investigate the effect of different incubation temperatures on lipase production by *P. candidum* PCA 1/TT031, separate tests were conducted under temperatures ranging from 4°C to 40°C.

The effect of different extractants on lipase production

In order to study the effect of sterilized extractants on lipase production, the crude extract of mouldy wheat bran was harvested using different extraction solutions. The mouldy bran was extracted by the addition of 50 mL sterilized 1% NaCl, 50 mM potassium phosphate pH 6.0-8.0, 50 mM Tris-HCl pH 9.0, 50 mM glycine-NaOH pH 10.0 and distilled water. Overnight soaking was allowed at a temperature of 4°C. The filtrate extracts were then assayed for lipase activity (Macedo *et al.*, 1997) and expressed as U/g WB.

Lipase assay through the oil emulsion method

This method, which was introduced by Macedo *et al.* (1997), entails the use of olive oil as the substrate for measuring lipase activity. One unit (U) of enzyme activity was expressed as the amount of 1 μmol of free fatty acid released per minute per mL extract.

Statistical analysis

As all the SSF experiments and analyses were performed in triplicate, the results are represented as their average \pm standard deviation. One way ANOVA using Minitab Software version 16 (Microsoft, USA) was performed for the statistical analysis, while Turkey's multiple range tests was employed to bring about the significant variation among means. 'Significant' was defined at the level of $p \leq 0.05$.

Results and discussion

The effect of different incubation times on lipase production

With this system, lipase production, which began on the third day and ended on the ninth day, was found to be constant and long lasting. As illustrated in Figure 2a, lipase production through *P. candidum* PCA1/TT031 increased substantially from the third day (0.7 U/g WB) to the seventh day (3.06 U/g WB). Subsequently, lipase production decreased gradually until the ninth day (1.3 U/g WB). This can be attributed to the complete consumption of the biodegradable fats (Santis-Navarro *et al.*, 2011). This also indicates that the lack of improvement in enzyme yields can be put down to the prolonged incubation period. This situation could be due to the secretion of proteases by the fungus which led to the cleaving and inactivation of the lipase (Diaz *et al.*, 2006). Wheat bran was used as a substrate for the time course study of lipase production. The results revealed that enzyme activity was at its peak on the fifth day of the fermentation process (Mahadik *et al.*, 2002).

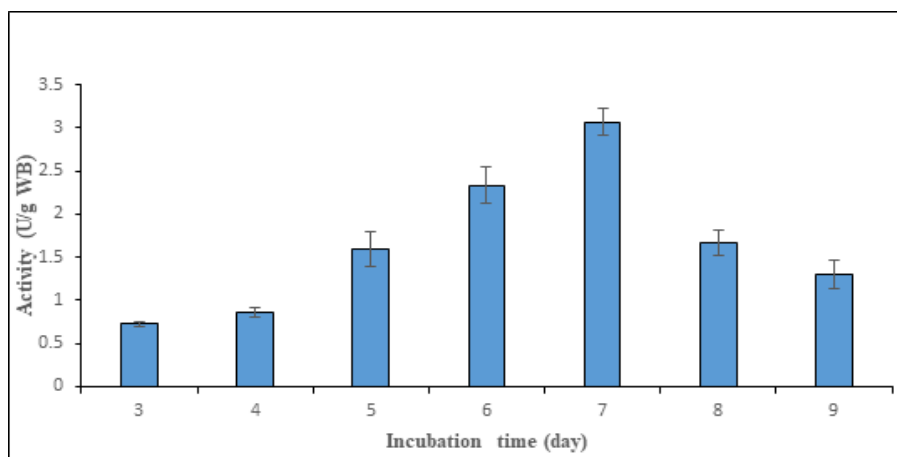


Figure 2a: Effect of incubation time on lipase production by *P. candidum* PCA 1/TT031.

Experimental condition: Wheat bran was incubated at 3, 4, 5, 6, 7, 8 and 9 days, moisture content 65%, inoculum size 1×10^6 spore/g WB, incubation temperature 25°C and extractant by Distilled water.

Note: Error bars denote the standard deviation from the mean of three independent experiments.

Oliveira *et al.* (2016) used a mixture of olive pomace to realize an increased production of *Aspergillus ibericus* lipase (90.5 ± 1.5 U/g) with a wheat bran ratio of 1:1, absence of Czapek nutrients, 60% of MC, $0.02 \text{ g NaNO}_3 \text{ g}^{-1}$ dry substrate and an incubation period of 7 days at a temperature of 30°C. Malilas *et al.* (2013) employed wheat bran as the substrate for the cultivation of *P. camembertii* KCCM 11268 in SSF for lipase production. By maintaining optimised conditions, maximum lipase activity was realized for eight days after fermentation. Thus, it can be deduced that by performing the roles of physical support and source of nutrients, SSF significantly promotes the levels of stability and activity of enzymes (Pandey *et al.*, 2000).

The effect of different carbon sources on lipase production

Carbon represents a critical nutrient and energy source for cells. It has been established, that the influence of various carbon sources on the production of extracellular enzymes through different strains, vary from one to another (Carrillo-Sancen *et al.*, 2016). The effects of compounds such as olive oil, starch, triton x-100, tributyrin, glucose and maltose at a concentration of 1% (w/w) on lipase production through *P. candidum* PCA1/TT031 were analysed. Table 1 lists the lipase production capacity of *P. candidum* PCA 1/TT031 in relation to different carbon sources. Interestingly, all the carbon sources were observed to elevate the production of lipase. The highest significant lipase production (4.3 U/g WB) was realized with the use of 1% tributyrin. With this study, a revised concentration of the best carbon source (1% tributyrin) was employed. A significant impact ($p \leq 0.05$) on lipase production through *P.*

candidum PCA 1/TT031 was detected with the use of 2% tributyrin. However, the enzyme production was observed to decrease with an increase in concentration beyond 2% (Figure 2b). This is in agreement with the findings by Maliszewska and Mastalerz (1992). They declared tributyrin to be the best substrate for extracellular lipase production through *P. citrinum* in SmF. For lipase production from *P. camembertii* in SmF, 1% olive oil proved to be a good inducer. Glucose, on the other hand, is considered a bad inducer (Amrane *et al.*, 2003).

Table 1: Effect of different carbon sources on lipase production by *P. candidum* PCA 1/TT031

1% Carbon source	Activity (U/g WB)
Starch	2.7 ± 0.1^{bc}
Olive oil	3.2 ± 0.2^b
Tributyrin	4.3 ± 0.1^a
Triton x-100	2.7 ± 0.2^{bc}
Glucose	2.4 ± 0.2^c
Maltose	2.3 ± 0.3^c

Note: Means with the same letter are not statistically different ($p > 0.05$)

The effect of different nitrogen sources on lipase production

Nitrogen sources, including inorganic and organic nitrogen, play a crucial role in the synthesis of enzymes. While inorganic nitrogen sources can be readily used, organic nitrogen acts as a source for the generation of amino acids and cell growth. These are crucial factors when it comes to enzyme synthesis and cell metabolism (Malilas *et al.*, 2013a). Generally, the production of lipases through microorganisms can be enhanced by employing a high concentration of nitrogen sources in the media (Mahanta *et al.*, 2008). Table 2 portrays the effect

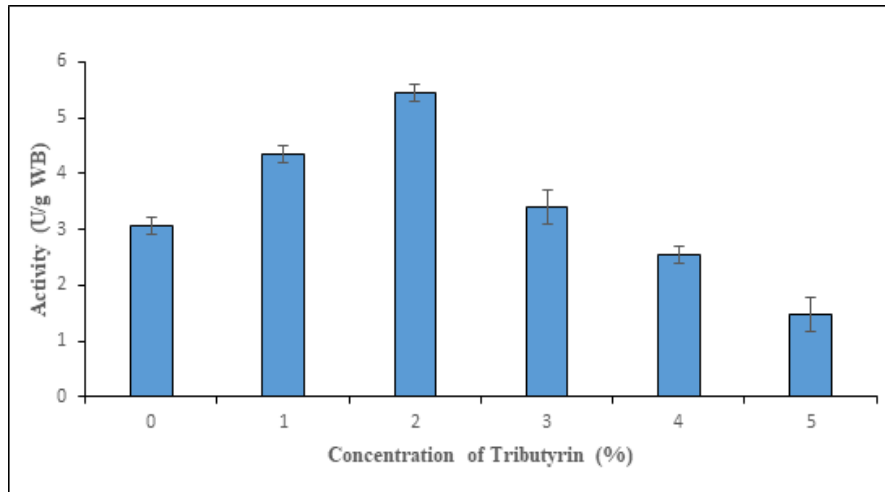


Figure 2b: Effect of different concentration of tributyrin on lipase production by *P. candidum* PCA 1/TT031

Experimental condition: Wheat bran was supplemented with 0, 1, 2, 3, 4 and 5% of tributyrin, moisture content 65%, inoculum size 1×10^6 spore/g WB, incubation temperature 25°C, incubated for 7 days and extractant by Distilled water.

Note: Error bars denote the standard deviation from the mean of three independent experiments.

of supplementation when different nitrogen sources are used for the production of lipase. Casein, yeast extract, meat peptone, $(\text{NH}_4)_2\text{SO}_4$, urea and NaNO_3 were employed as organic and inorganic sources for the provision of additional nitrogen. The results revealed meat peptone to be the best nitrogen source. Its supplement led to a 4.7 U/g WB climb in lipase production. Although wheat bran comes with a good amount of crude protein, the use of supplemented nitrogen for enhanced access served to further improve enzyme production. Different concentrations (0 to 5%) were employed to study the effect of varying concentrations of meat peptone on the activity of the lipase. The highest significant activity (5.2 U/g WB) was achieved when 2% of meat peptone was used to produce extracellular lipase by *P. candidum* PCA 1/TT031 (Figure 2c). Kaushik *et al.* (2006) employed meat peptone for lipase production in submerged fermentation by using *Aspergillus carneus*, while Teng and Xu (2008) and Wang *et al.* (2008) employed *Rhizopus chinensis*. Mahadik *et al.* (2004) made use of a combination comprising yeast extract and peptone to produce lipase through *A. niger*. Colla *et al.* (2016) realized maximum lipolytic activities by employing 45 g/L of yeast extract at pH 7.15. Here, wheat bran was used as the substrate for filamentous fungi. The use of organic nitrogen as sources for the production of lipases through fungi has been reported by several researchers. Miranda *et al.* (1999) examined the production of lipases using oil refinery waste containing nitrogen sources such as urea, ammonium chloride and ammonium sulphate. The use of *Antrodia cinnamomia* with ammonium chloride provided the best results.

Table 2: Effect of different nitrogen sources on lipase production by *P. candidum* PCA 1/TT031

1% Nitrogen source	Activity (U/g WB)
Casein	2.4±0.3 ^c
Meat peptone	4.7±0.2 ^a
Yeast extract	3.8±0.1 ^b
Urea	2.4±0.3 ^c
$(\text{NH}_4)_2\text{SO}_4$	1.7±0.2 ^d
NaNO_3	2.0±0.2 ^{cd}

Note: Means with the same letter are not statistically different ($p > 0.05$)

The effect of inoculum size on lipase production

The size of inoculum is important as it a decisive factor in the production of biomass during the fermentation process. As such, the mould size needs to be regulated to ensure a high production of enzymes in the limited weight of the substrate. It was observed that the inoculation of different production media employing different inoculum sizes (from 1×10^6 to 1×10^7 spore/g WB of *P. candidum* PCA 1/TT031) had varying effects on lipase production. As shown in Figure 2d, the maximum lipase production was achieved with an inoculum size of 5×10^6 spore/g WB (11.3 U/g WB). Bigger inoculum sizes resulted in a decreased lipase production. This could be attributed to the dearth of nutrients that can be secured by a large biomass for faster culture growth (Maia *et al.*, 2001). Hence, maximum enzyme production can be achieved by striking a balance between the available materials and the proliferating biomass. The lipase activity revealed the existence of significant statistical disparities between the various inoculum sizes in the mouldy bran ($p \leq 0.05$). The rise in lipase production, due to the employment of small inoculum sizes, is probably attributed to the

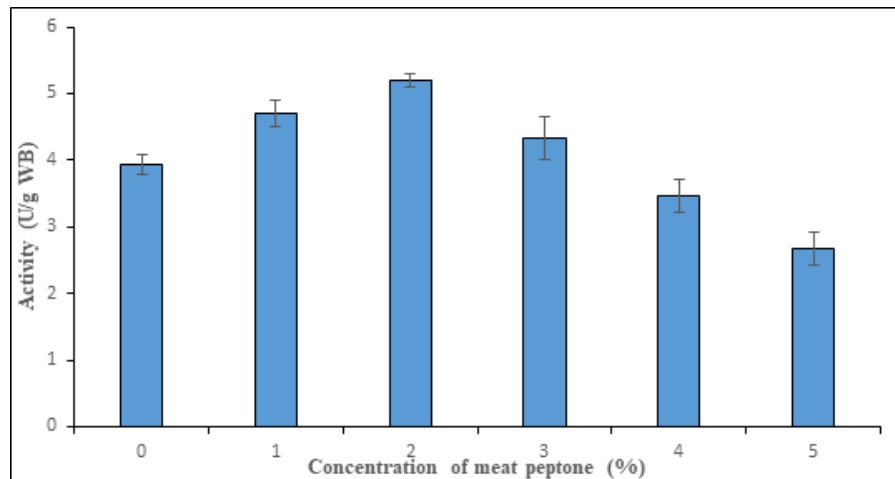


Figure 2c: Effect of different concentration of meat peptone on lipase production by *P. candidum* PCA 1/TT031

Experimental condition: Wheat bran was supplemented with 0, 1, 2, 3, 4 and 5% of meat peptone, tributyrin 2%, moisture content 65%, inoculum size 1×10^6 spore/g WB, incubation temperature 25°C, incubated for 7 days and extractant by Distilled water.

Note: Error bars denote the standard deviation from the mean of three independent experiments.

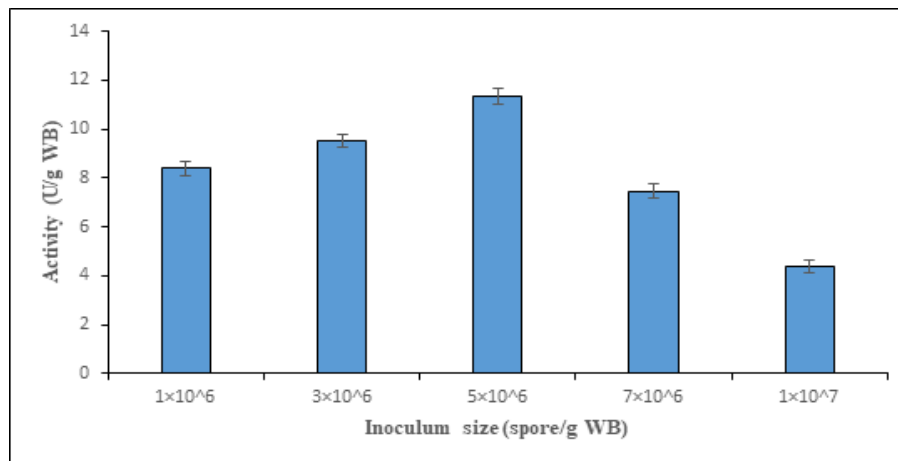


Figure 2d: Effect of different inoculum size on lipase production by *P. candidum* PCA 1/TT031

Experimental condition: Wheat bran was cultivated with from 1×10^6 , 3×10^6 , 5×10^6 , 7×10^6 and 1×10^7 spore/g WB, tributyrin 2%, meat peptone 2%, moisture content 50%, initial pH 9.0, incubation temperature 25°C, incubated for 7 days and extractant by Distilled water

Note: Error bars denote the standard deviation from the mean of three independent experiments.

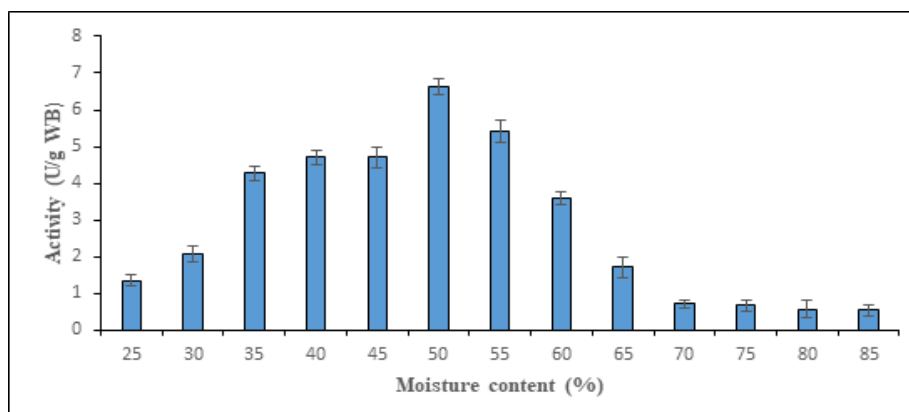


Figure 2e: Effect of different moisture content on lipase production by *P. candidum* PCA 1/TT031

Experimental condition: Wheat bran was wet with 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 85% of distilled water, tributyrin 2%, meat peptone 2%, inoculum size 1×10^6 spore/g WB, incubation temperature 25°C, incubated for 7 days, and extractant by Distilled water

Note: Error bars denote the standard deviation from the mean of three independent experiments.

raised ratio between the surface area and the volume. However, it was observed that lipase production was reduced with a higher inoculum of 5×10^6 spore/g WB. Generally, this yield was more than that realized with the lower inoculum size of 1×10^6 spore/g WB. Thus, high inoculum sizes need not necessarily lead to a greater lipase yield. Indeed, high inoculum sizes may lead to a depletion in oxygen and nutrients in the culture media (Abol Fotouh *et al.*, 2016). ul-Haq *et al.* (2002) examined the effect of various inoculum sizes of *Rhizopus oligosporus* GCBR-3 on lipase production under SSF. An inoculum size of 1.0% provided the maximum production (48 U/g substrate). Higher enzyme activity was not supported by either higher or lower inoculum sizes. A 5% inoculum size of *Rizopus oligosporus* DGM 31 used by Iftikhar *et al.* (2008) gave rise to the maximum intracellular lipase production. This could be due to the fact that an optimum amount of enzymes is produced by an optimum level of mycelium, for which inoculum of 1.0 mL is deemed more than sufficient. Other researchers have reported different optimum inoculum sizes for different microorganisms. An ideal value would be a 1% spore suspension ($\sim 10^8$ cells/mL) size for *Aspergillus* TL-12 inoculum to realize an improved lipase production (Tagore and Narasu, 2012). The cultivation of 2 mL inoculum of *Penicillium fellutanum*, with canola seed oil cake as the substrate at a temperature of 30°C, led to a maximum lipase production (Amin and Bhatti, 2014). These findings indicate that both age and inoculum concentrations profoundly influence lipase production. Different inoculum sizes are required by different organisms to realize maximum lipase production.

The effect of different moisture contents on lipase production

Moisture content is considered a crucial factor during the production of enzymes. Fungus growth and enzyme biosynthesis are significantly influenced by [a] low and high-medium moisture contents (Wang *et al.*, 2006; Behnam *et al.*, 2016), [b] the physical properties of the solid substrate (Bakri *et al.*, 2003), as well as [c] the organism itself (Wong *et al.*, 2017). Therefore, it is crucial to determine the optimum moisture content (which is dependent on the kind of substrate end product), and the nutrients required by the fungus (Lonsane *et al.*, 1985). The impact of moisture content on the production of lipase production was examined within the range of 25% to 85%. As shown in Figure 2e, the maximum lipase production by *P. candidum* PCA 1/TT031 was realized at a wheat bran moisture content of 50% (6.6 U/g WB). It was observed that the enzyme activity

dipped to 0.5 U/g WB with a further increase in the substrate's moisture level content. A decrease in substrate porosity due to a high water content (which may be linked to the reduction in the porosity of the medium), lowers the oxygen transfer rate. This situation leads to the creation of an unfavourable environment for fungi growth. Conversely, a low water availability level gives rise to issues that include high water tension, substrate swelling, as well as reduced nutrient transportation and diffusion (Mahadik *et al.*, 2002; Wong *et al.*, 2017). A high moisture content level facilitates earlier enzyme production and quicker fungal growth. These findings are in line with those associated to Amin and Bhatti (2014). They employed canola seed oil cake containing 50% moisture as the substrate to realize maximum lipase production (521 U/g dry substrate) from *P. fellutanum*. A similar finding was reported by Kempka *et al.* (2008). They achieved maximum lipase production by employing *Penicillium verrucosum* under optimised initial moisture of the substrate (55%), and a cultivation incubation temperature of 27.5°C. The optimum wheat bran moisture content for cultivated *P. camembertii* KCCM 11268 was studied by Malilas *et al.* (2013). They discovered the optimum initial moisture content of wheat bran to be 60% for the production of lipase. The maximum enzyme yield for *A. niger* NCIM 1207 was determined through the moistening of wheat bran with a synthetic oil-based (SOB) medium in a 1:2.5 ratio.

The effect of different initial pH on lipase production

A factor that can considerably influence the growth of fungi during solid SSF is pH (Mahanta *et al.*, 2008; Ire *et al.*, 2011). The transportation of different components across cell membranes and various enzymic processes is significantly affected by the pH of the medium (Sandhya *et al.*, 2005). The pH value of the culture media controls the synthesis of extracellular enzymes through different types of microorganisms (Wong *et al.*, 2017). The fermentation media's pH value may also undergo modification during the fermentation process. This is due to the fact that the used substrates employed in SSF generally come with the least buffering. As changes in pH have the least effect on lipase production, differential initial pH levels ranging from 6.0 to 12.0 was considered for tests involving wheat bran. As exhibited in Figure 3, on the seventh day of the fermentation process, the initial pH value of 9.0 was revealed to be most ideal for lipase production by *P. candidum* PCA 1/TT031 (8.6 U/g WB). However, it is notable that lipase activity was apparent throughout the pH range (6.0 to 12.0) tested

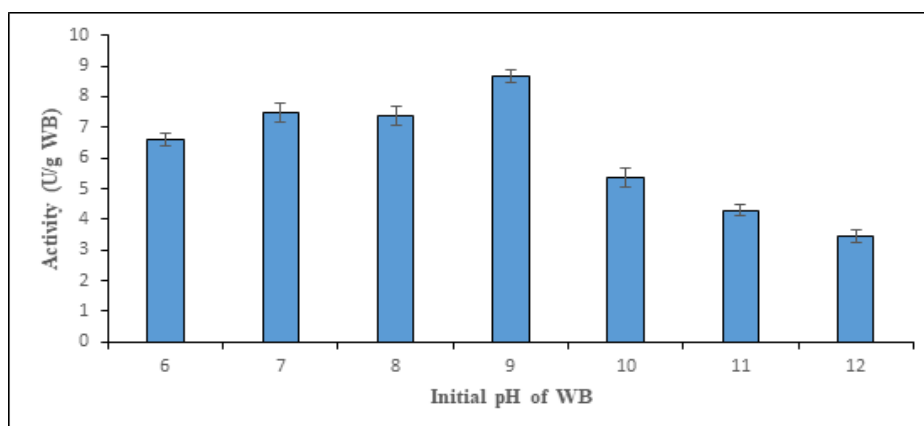


Figure 3: Effect of initial pH wheat bran of on lipase production by *P. candidum* PCA 1/TT031

Experimental condition: Wheat bran was adjusted to 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0, tributyrin 2%, meat peptone 2%, moisture content 50%, inoculum size 1×10^6 spore/g WB, incubation temperature 25°C, incubated for 7 days, and extractant by Distilled water.

Note: Error bars denote the standard deviation from the mean of three independent experiments.

during this investigation. The intracellular pH of cells can be enhanced through alkaline extracellular pH, as well as through the facilitated destabilisation of the enzyme synthetic network. This is attributed to the fact that at specified intracellular pH values, equilibrium can be realized for enzyme catalysed reactions (Von Stockar *et al.*, 2006), as well as for the intracellular ionic strength of the biological system (Iftikhar *et al.*, 2008). The initial pH of the medium was observed to be significantly ($p \leq 0.05$) affected by the lipase production through the organism. This was established through a scrutiny of the variance results. Ortiz-Vazquez *et al.* (1993) reported the optimum initial pH of wheat bran for lipase production from *P. candidum* to be 7.0. The initial pH of the cultivation medium ultimately determines if the end-product is acidic or alkaline in nature (Wong *et al.*, 2017). The maximal yield of lipase by *Saccharomyces lipolytica*

was detected at pH 9.5 (Muralidhar *et al.*, 2001).

The effect of different incubation temperatures on lipase production

Temperature is an environmental factor that can significantly influence lipase production through microorganisms (Krishna, 2005). *P. candidum* PCA 1/TT031 was employed for an examination on various temperatures (4, 15, 20, 25, 30 and 40°C) to determine the optimum temperature for lipase production. The optimum temperature for mouldy bran was revealed to be 20°C for lipase production (13.6 U/g WB) (Figure 4). An incubation temperature beyond 20°C led to a significant dip in lipase production (0.26 U/g WB at 40°C. According to Palma *et al.* (2000), this can be attributed to the increase in protease production which led to the deactivation of lipase when the incubation temperature breached 20°C. The increase

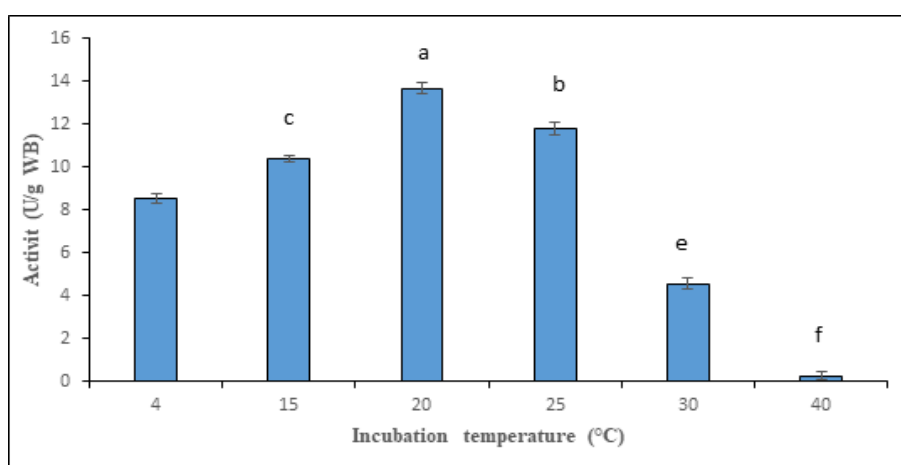


Figure 4: Effect of different incubation temperature on lipase production by *P. candidum* PCA 1/TT031

Experimental condition: Wheat bran was incubated at 4, 15, 20, 25, 30 and 40°C, tributyrin 2%, meat peptone 2%, moisture content 50%, initial pH 9.0, inoculum size 5×10^6 spore/g WB, incubated for 7 days and extractant by Distilled water.

Note: Error bars denote the standard deviation from the mean of three independent experiments.

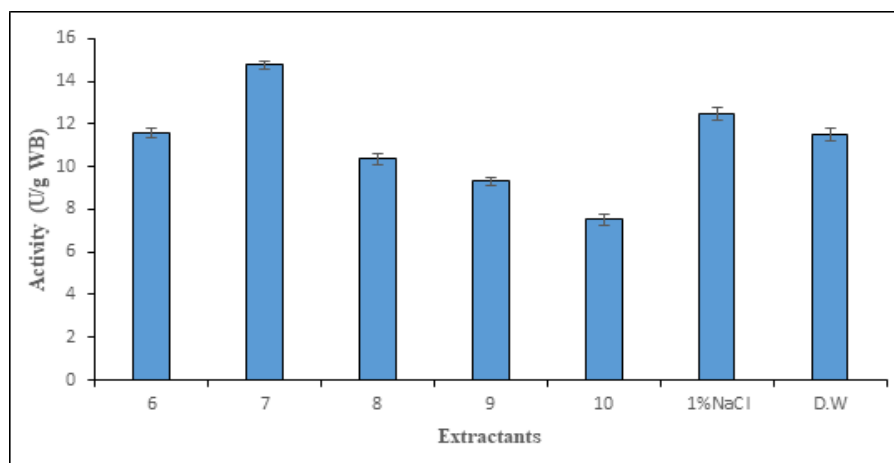


Figure 5: Effect of different extractants solution on lipase production by *P. candidum* PCA 1/TT031

Experimental condition: Wheat bran was extract using solution from pH 6.0 to 10.0, Distilled water and 1% NaCl, tributyrin 2%, meat peptone 2%, moisture content 50%, initial pH 9.0, inoculum size 5×10^6 spore/g WB, incubation temperature 20°C, incubated for 7 days.

Note: Error bars denote the standard deviation from the mean of three independent experiments.

in incubation temperature also led to a substantial dip in the moisture of wheat bran. This could be due to the higher water uptake rate of the microorganism, and the rise in water vapour pressure in the system upon exposure to raised temperatures (Parihar, 2012). Ortiz-Vazquez *et al.* (1993) employed wheat bran at 29°C to realize the highest lipase activity for *P. candidum* on the eighth day. Kempka *et al.* (2008) incubated *P. verrucosum* with soybean bran as the substrate at 27.5°C to arrive at the highest lipase activity (40 U/g of dry bran).

The effect of extractants on lipase production

50 mL of sterilised 1% NaCl, 50 mM Tris-HCl pH 9.0, 50 mM potassium phosphate pH 6.0 to 8.0, distilled water and 50 mM glycine-NaOH pH 10.0 were employed as the extractant in the ratio of 5 mL/g WB to examine the influence of the extractant's pH. Distilled water represented the control. According to the results displayed in Figure 5, the type of extractant used, and the pH level, had a significant impact on the extraction process when compared to the use of distilled water as the extractant. The maximum lipase production (14.7 U/g WB) was achieved through a phosphate buffer of pH 7.0 with the mouldy bran, and then with 1% NaCl (12.5 U/g WB). This is in agreement with the findings of Mahadik *et al.* (2002) who used *Aspergillus niger* to realize maximum lipase activity. This was achieved through the extraction of fermented wheat bran with NaCl (1%) and a Triton X-100 (0.5%) supplement. Santis-Navarro *et al.* (2011) discovered that lipases extracted from a mixture of wastewater sludge are most active when the extracting conditions are maintained at a temperature of between 61°C to

65°C, and a pH level within 7.7 and 9.0.

Conclusions

The steps involved in the use of wheat bran as a substrate by *P. candidum* PCA 1/TT031 for the extraction optimisation of extra-cellular crude lipase, significantly influences the subsequent purification steps. Thus, different factors were individually studied to uncover the ideal cultivation condition for the production of lipase. The peak activity of enzyme production was achieved after 7 days of incubation with *P. candidum* PCA 1/TT031. The highest lipase activity was realized with 2% tributyrin through the addition of different carbon sources. Subsequent to the screening of different inorganic and organic nitrogen sources, it was revealed that lipase production significantly escalates with 2% meat peptone. Investigations also revealed that lipase production can be enhanced by maintaining a moisture content of 50%, an initial pH of 9.0, an inoculum size of 5×10^6 spore/g WB, and a mouldy bran incubation temperature of 20°C.

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