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Production of fungal amylase and cellulase enzymes via solid state fermentation using *Aspergillus oryzae* and *Trichoderma reesei*

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Abstract

Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial applications. The process optimization of two fungal enzymes amylase and cellulase was done using two non toxic fungi *Aspergillus oryzae* and *Trichoderma reesei* fermenting deoiled rice bran in the solid state. Various optimized cultural conditions were a temperature of 30°C, 75% moisture content , pH 5.5, spore suspension $1x10^7$/ml and incubation period of 5 days. Under all optimized conditions the deoiled rice bran yielded an amylase activity of 0.787 IU and cellulase activity of 0.587 IU.

Keywords: *Aspergillus oryzae, Trichoderma reesei, cellulase, amylase, deoiled rice bran, SSF.*

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1. Introduction

Solid State Fermentation (SSF) has been exploited for the production of feed and food. It is a technology which uses a reduced reactor volume per unit of converted substrate, where fungi are applied to obtain the desired product [1]. This technology usually uses agro-industrial waste as support and carbon source for production of various value added products, such as single cell protein, industrial enzymes, secondary metabolites and fine chemicals [2]. Agro-industrial residues are generally considered the best substrates for the SSF processes. A number of such substrates have been employed for the cultivation of microorganisms to produce host of enzymes [3]. Compared to submerged fermentation, the SSF process is a simple process with improved product characteristics, higher product yields, reduced energy requirements and initial capital cost, lower water output and easier product recovery. It has been reported that it is the most appropriate process in developing countries due to the advantages it offers [4] . Solid state fermentation appears to possess several biotechnological advantages, such as higher fermentation productivity, higher end concentration of products, higher product stability, lower catabolic repression, mixed cultivation of various fungi and lower demand on sterility due to the low water activity used in SSF [5] . In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities, thus optimum moisture level of the substrate is the most important factor for enzyme production [6]. Solid-state fermentation has gained renewed interest and fresh attention from researchers owing to its importance in recent developments in biomass energy conservation, in solid waste treatment and in its application to produce secondary metabolites [7]. Use of suitable low cost

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fermentation medium for production of alpha amylase using agricultural by-products has been reported [8].

India is an agrarian country where two-third of its population depends on rice as staple food. One of the major primary byproducts of rice milling is rice bran which accounts for 8% of the milled rice [9]. Rice bran is a source of proteins, oils and nutrients. Raw rice bran contains about 18 to 20% oil whereas parboiled rice bran contains about 22 to 25% oil. Currently, 60% of the rice bran produced is used as animal feed, while the rest of the 40% is used to produce value added edible cooking oil. On an average, 4 million tonnes of rice bran, 0.6 million tonnes of rice bran oil and 0.2 million tonnes of deoiled rice bran waste are generated annually. The deoiled rice bran waste contains 39% of cellulose and 9% of protein [10]. During rice bran oil processing, a large amount of bran nutrition is lost along with the byproducts. There are two major byproducts of rice bran oil processing, deoiled rice bran cake and soap stock. The deoiled bran, which is a rich source of protein (about 17%) and vitamin B, is used as cattle feed and poultry feed. Hence, the present study was aimed to accomplish the Solid state fermentation (SSF) of rice bran deoiled cake by co-cultures of *Aspergillus oryzae* and *Trichoderma reesei*.

2. Materials and Methods

2.1. Procurement and maintenance of cultures

Two non-toxic fungi, *Aspergillus oryzae* for amylase production (MTCC 3107) and *Trichoderma reesei* (MTCC 164) for cellulase production were procured from MTCC (Microbial Type Culture Collection), Institute of Microbial Technology, Chandigarh, Punjab. Both these cultures are Generally Recognized as Safe (GRAS) [11], [12], [13], [14],[15]. Both the cultures were maintained by sub-culturing fortnightly on Potato Dextrose Agar (PDA) slants and were stored subsequently at 4°C in a refrigerator.

2.2. Mineral media for enzyme production

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The composition of mineral medium was the one given by Singh and Surendra [16].

2.3. Solid state fermentation for the production of fungal enzymes , enzyme extraction and enzyme assays

2.3.1. Preparation of Inoculum

A spore inoculum was prepared by adding 10-15 ml of sterile Tween 80 (0.8%) to each slants having the fungal cultures and shaken vigorously. One ml of the inoculum from the fungal slants of both the cultures was used per flask to carry out solid state fermentation of deoiled rice bran.

2.3.2.. Enzyme production by solid state fermentation

Five gram of unfermented deoiled rice bran was taken in individual Erlenmeyer flask (250 ml) with 15 ml of mineral medium i.e. 1:3 ratio [17] and autoclaved at a pressure of 1.1 kg/cm^2 for 20 minutes. The flasks were cooled to room temperature and inoculated with one ml of the spore suspension of both the cultures. The inoculated flasks were then incubated at $28 \pm 2^{o}C$ for five days in a BOD incubator in static condition.

2.3.3. Extraction of enzyme

After fermentation, the flasks were taken out and brought to room temperature. The product was recovered from the substrate by shaking it for 30 min in shaking incubator (250 rpm) with 0.1M citrate buffer at a solid to moistening agent ratio of 1:10. The extract was then, filtered through Whatman No. 1 filter paper to obtain a clear filtrate. The filtrate was then centrifuged at 5000 rpm for 20 minutes. The supernatant obtained was again filtered through Whatman filter paper No. 1 so as to obtain a cell free supernatant which was used as a source of crude enzyme [18]. Alpha amylase and cellulase activity were estimated by spectrophotometric method.

2.3.4. Enzyme assays

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The activities of cellulase and amylase enzyme were expressed in International Units (IU). One IU is defined as one µmol of glucose (for amylase and cellulase activity) equivalents released per minute per ml under the assay conditions by using glucose standard curve [19]. Appropriate dilution factors were used during the estimation of enzyme activity.

2.3.4.1. Alpha amylase activity

Alpha amylase activity was determined according to the method reported by Miller [20].

2.3.4.2. Cellulase activity

Cellulase activity was estimated as Carboxy Methyl Cellulase (CMCase) according to the method given by Ray *et al* [21] .

3. Results and Discussions

The present study was carried out to study the production of fungal enzymes viz. amylase and cellulase using two different hydrolytic fungi viz; *Trichoderma reesei* (for cellulase production) and *Aspergillus oryzae* (for amylase production) together.

3.1 Effect of different cultural conditions on production of enzymes

3.1.1 Effect of initial moisture content on production of enzymes

Varying amounts of mineral medium were added to the substrate, to study the effect of moisture content on enzyme production. The results illustrated an increase in the alpha amylase and cellulase activities with increase in mineral medium level from 10 to 20 ml/ 5 g substrate and with further increase in moisture content up to 30 ml/ 5 g substrate, lead to a decrease in alpha amylase activity and cellulase activity as depicted in Fig 1(a). Hence 15 ml medium/ 5 gm substrate i.e. 1:3 substrates to medium ratio was regarded as the optimum moisture level. Moisture is the key element for regulating and optimizing the solid state

Fig 1(a) Effect of moisture content on production of fungal enzymes

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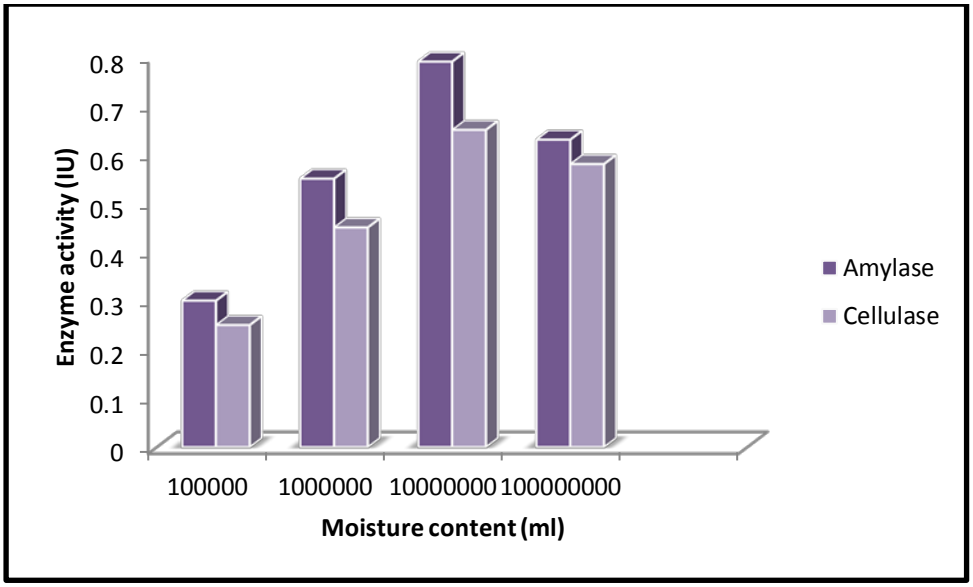

fermentation process. Intermediate moisture content is required for an efficient solid state fermentation process [22]. In a similar study by Chimata *et al* [23], highest amylase activity of 164U/g at 70% moisture level as compared to 80% moisture level, was reported using wheat bran as substrate which was fermented with *Aspergillus sp*. MK07. On the contrary, Kahlon and Das [24] observed that the ratio for the optimum moisture content is 1:4 (40ml/10gm) of rice straw fermented in the solid state by *Pleurotus ostreatus*.

Kundu *et al* [25] observed that below the determined optimal moisture level for solid state culture, there was enzyme inhibition and above the optimum level, there was greater enzyme diffusion away from the substrate. Laukevics *et al* [22] also observed that very little moisture inhibits the growth and enzyme activity of the fungi and also the accessibility to nutrients, while very high moisture compacts the substrate, prevents oxygen penetration and facilitates contamination by fast growing bacteria. Fungus grows at lower water ratio, which offers significant advantage in reducing the risk of contamination; since most bacterial species are unable to grow at reduced moisture level [18]. However, the optimum moisture level varies with the substrate used, as different types of substrate have different water holding capacity.

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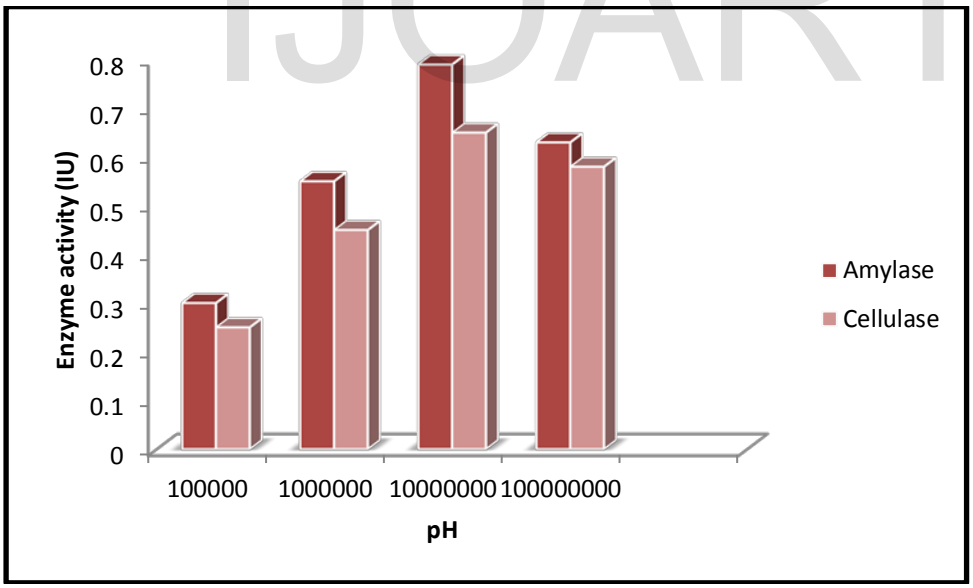
3.1.2 Effect of initial pH on enzyme production

The results depicted in Fig 1(b) indicate that with increase in pH value from 4.0 to 5.5, the activity of amylase and cellulase enzymes reached to the maximum followed by a gradual decrease thereafter. Optimum pH for both amylase and cellulase activity was 5.5 and their activity reduced to 50% when pH was increased from 5.5 to 6.0. Change in pH from the optimum to extreme levels results in inactivation of the enzymes of the organisms which hinder saccharification of the substrate. Similar results were reported by Silva *et al* [19], who reported that the optimum pH for CMCase production was 5.5 using *Thermoascus aurantiacus* and their activity dropped to 50% when pH was increased to 6.5. Gomes *et al* [26] also reported that optimum pH for the crude CMCase production was 5.5 using *Thermoascus aurantiacus.*

Fig 1(b): Effect of pH on production of fungal enzymes



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Solid state fermentation of *Aspergillus oryzae* for amylase production on agro residues was studied by Zambare [27], who reported an optimum amylase production of 0.198 IU using dry fermented wheat bran at pH 6.0. Behaviour of novel thermostable β-amylase from *Clostridium thermosulfurogenes* on raw starch was studied and optimum pH for raw starch hydrolysis was reported to be 4.5 - 5.5 [28]. Enzymes had optimal activities at pH values between 4.5 and 6.0 and were stable under pH range of 4.0-7.0 [29].

3.1.3 Effect of incubation temperature on enzyme production

The effect of temperature on amylase and cellulase activity of *Aspergillus oryzae* and *Trichoderma reesei* was studied by varying the temperature from 20°C to 40°C as depicted in the Fig 1(c). It is clear from the results that a temperature of 30°C was found to be best for amylase and cellulase activity. Lesser activity of fungal enzymes at low temperature (20-25°C) and at high temperature (35-40°C) as compared to 30°C might be due to slow growth at low temperature and inactivation of the enzyme at high temperature. In a similar finding by Gupta *et al* [30], the optimum temperature for protein production and extra cellular enzymes (cellulase) was 30°C using *Coprinus attramantarius*. Similarly, maximum amylolytic activity of thermophilic fungi *Aspergillus fumigates* isolated from soil was observed at 30°C in mineral medium containing 1% starch and 1.5% organic nitrogen concentration [31].

3.1.4 Effect of inoculum concentration on enzyme production

Proper amount of inoculum is essential for an efficient solid state fermentation. The results presented in Fig 1 (d) showed that with the increase in inoculum size from 1×10^5 to

Fig 1(c): Effect of incubation temperature on production of enzymes

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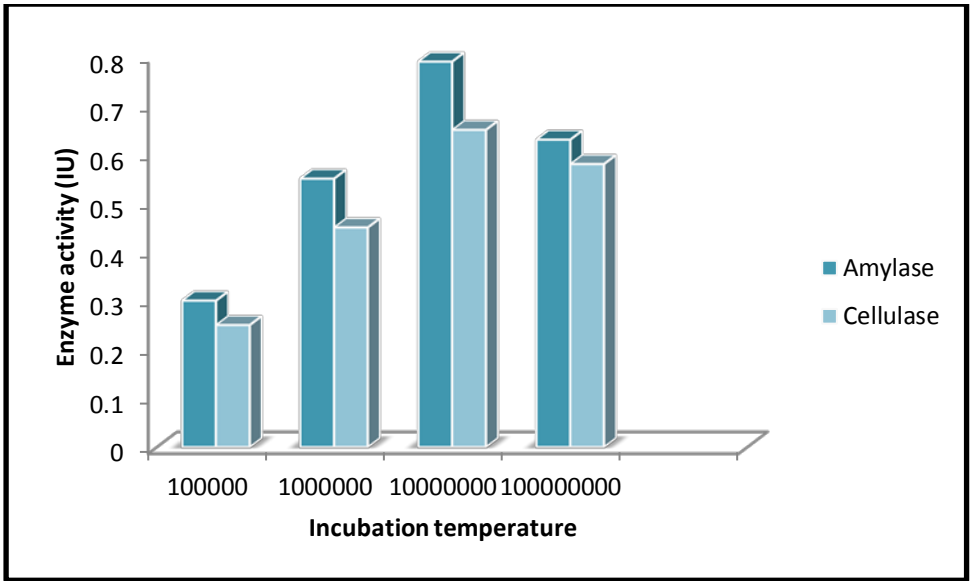
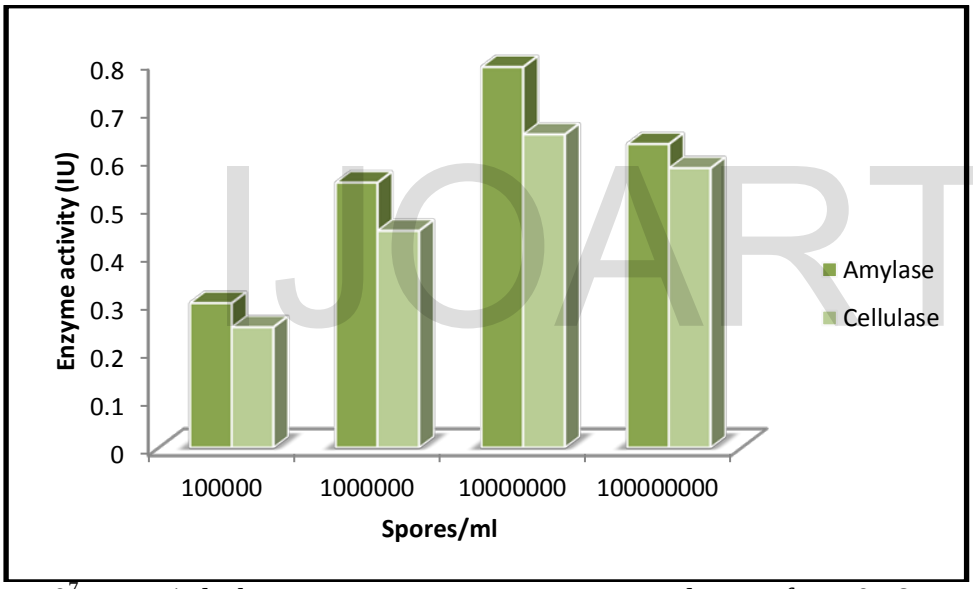


Fig 1(d) : Effect of inoculum concentration on production of enzymes



1×10^7 spores/ml, there is an increase in enzyme production from 0.281 to 0.734 IU of alpha amylase and 0.237 to 0.541 IU of cellulase respectively. Thus, inoculum size of 1×10^7 spores/ml was optimum for maximum enzyme activity. Similar results were reported by Murthy *et al* [32], who used coffee industry substrates for the synthesis of alpha amylase by solid state fermentation using a fungal strain of *Neurospora crassa* CFR 308. Maximum alpha amylase activity was obtained using coffee pulp as substrate with a spore suspension of $1x10^7$ spores/ml.

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An increase in the number of spores in the inoculum would ensure rapid proliferation and biomass synthesis. A higher inoculum size may increase moisture content and lead to a decrease in growth and enzyme production. A lower inoculum size may require a longer time for fermentation to form the desired product [6]. Inoculum level of 10% (v/w) at pH 6, temperature 50°C and initial moisture content of 90% was optimum for alpha amylase activity obtained by *Thermomyces lanuginosus* fungus on wheat bran [33]. On the contrary, Pankaj and Satyanarayana [34] observed maximum cellulase and xylanase production of 7832 U/g of dry moldy bran with an inoculum level of 3×10^6 spores of *Humicola lanuginose* / 10 g wheat bran by solid state fermentation.

3.1.5 Effect of incubation period on enzyme production

Incubation period plays an important role in substrate utilization and its protein enrichment for enzyme production. It was found that the maximum yield of alpha amylase activity (IU) and cellulase activity (IU) was observed on fifth day of incubation (Fig 1e). Similarly, Chimata *et al* [23] reported the production of extracellular amylases by solid state fermentation (SSF) employing a laboratory isolate *Aspergillus sp.* MK07 and found that amylase production (164 U/g) was highest on 5^{th} day of incubation period at 30°C. Similarly Kang *et al* [35], found highest cellulase activity after 5-6 days of fermentation by *A. niger* on rice straw.

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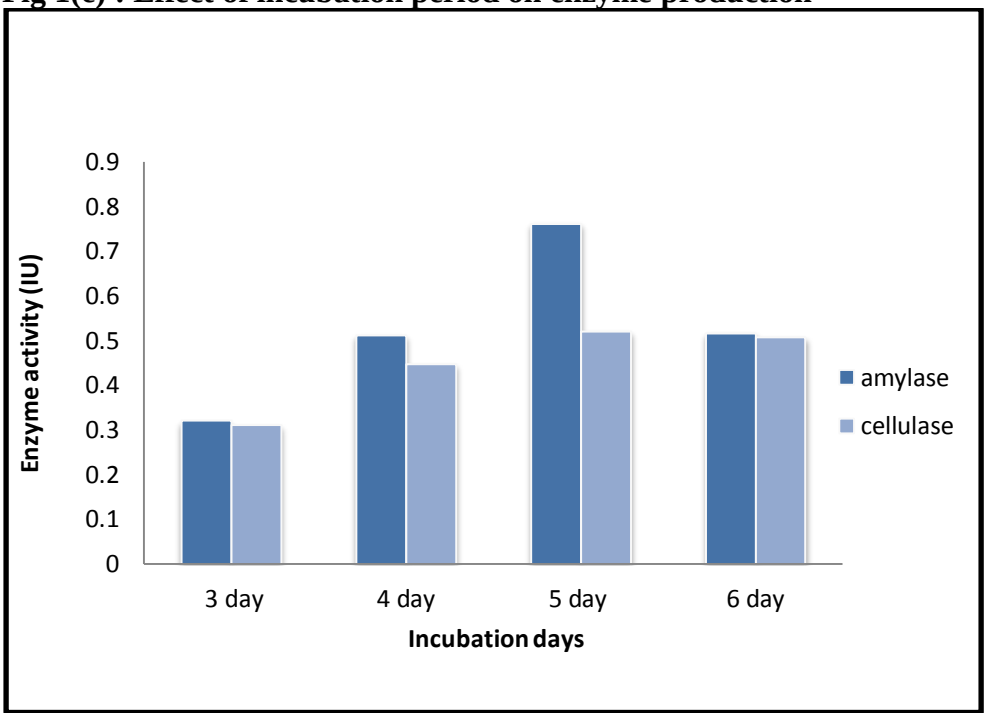
Fig 1(e) : Effect of incubation period on enzyme production



Enzyme production is related to growth of fungus, so the growth of the organisms might have reached a stage that indirectly stimulates production of secondary metabolites [36]. Solid-state cultivation of *Fusarium solani* was carried out for enhanced production of glucoamylase (GA) using different substrates like wheat bran, rice bran, green gram bran, black gram bran and maize bran. Maximum enzyme activity 61.35±3.69 U/g of dry wheat bran was achieved with the optimum incubation period of 120 hrs [37]. Thus, it was demonstrated that prolonged processing time had an adverse effect on the progress of the solid state fungal growth. Thus the optimum incubation time for maximum enzyme production depends on the type of media, growth rate of microorganism on particular carbon source and its enzyme production pattern.

3.1.6 Effect of nitrogen source on enzyme production

In addition to the carbon and energy source, microorganisms require nitrogen, phosphorus and minerals for their luxuriant growth. In our study, urea was a better nitrogen source for amylase and cellulase production by *Aspergillus oryzae* and *Trichoderma reesei* as

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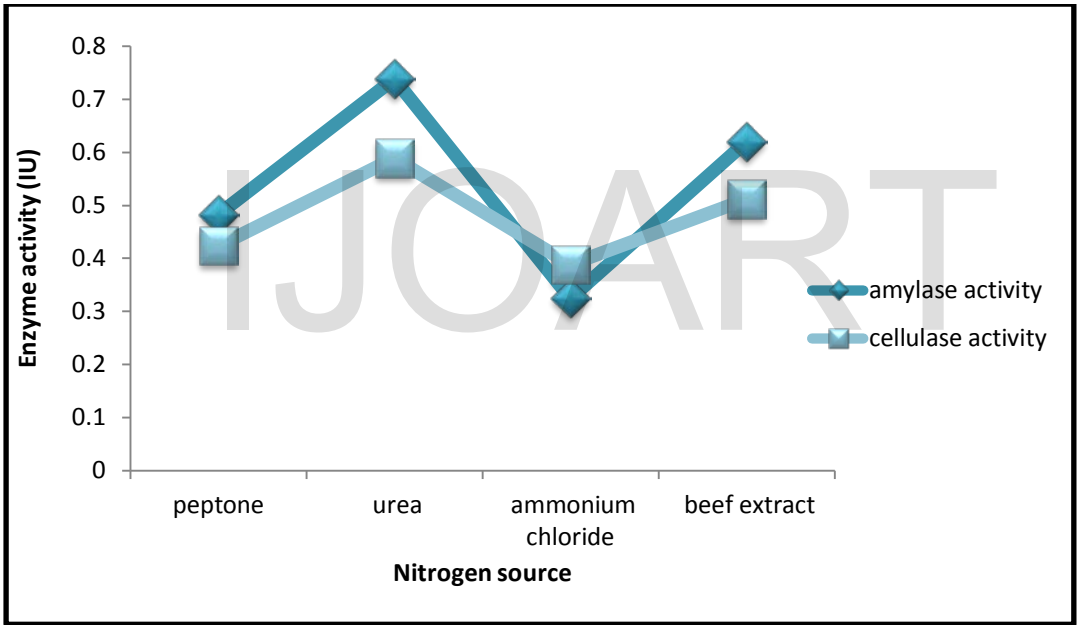
shown in Fig 1(f). It was observed that the maximum enzyme production occurred when urea was used as the nitrogen source followed by beef extract, peptone and ammonium chloride. According to Litchfield [38], the nitrogen sources found most suitable for SCP production are ammonia salts, urea, calcium ammonium nitrate, ammonium sulphate and potassium nitrate. Enzyme production is more in medium containing organic nitrogen sources, especially urea as compared to inorganic nitrogen sources [39]. In a similar study by Correa *et al* [40], urea was the best nitrogen source for highest cellulase activity for fermentation of sugar cane baggase with co cultures of *T. reesei* and *A. niger.*

Fig 1(e): Effect of nitrogen source on enzyme production



However, the study involving amylase production by *A. oryzae* on wheat bran indicated that none of the supplied organic nitrogen source showed any positive effect on the enzyme production, although all of them promoted good fungal growth. Some of the organic nitrogen sources (peptone and yeast extract) resulted in substantial reduction of enzyme yield as excess of complex nitrogen turned out to have an adverse effect on enzyme synthesis [41]. Likewise, an isolate of *A. flavus* from mangrove yielded the maximum amylase when grown

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on sugarcane baggase supplemented with yeast extract rather that tryptone, sodium nitrate, peptone, urea and ammonium sulphate [42].

4. Conclusion

Fungal enzymes amylase and cellulase were produced using a specific culture *Aspergilus oryzae* and *Trichoderma reesei*. Various parameters viz. temperature, pH, initial moisture, incubation time and inoculum concentration were studied to optimize the conditions to carry out SSF of rice bran deoiled cake by *Aspergillus oryzae* and *Trichoderma reesei.* The optimized cultural conditions for maximum production of enzyme were moisture content (1:3 i.e. 15ml/ 5g substrate), pH (5.5), temperature (30 °C), inoculum concentration (1 X 10^7 spores/ml) and incubation days (5 days). Hence from the present study we conclude that solid state fermentation of the deoiled rice bran cake for the optimal production of enzyme is technically feasible.

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