Chapter2: Utilization of starchy waste and optimization of physico-chemical process parameters for maximum acidophilus amylase production by *Aspergillus niger* RBP7 through solid state and submerged fermentation

2.1. Introduction

The uses of synthetic medium in fermentation is very expensive and uneconomical therefore, the production of amylolytic enzyme should be done using low cost substrates like different agricultural and agro- industrial wastes which minimizes the overall cost of production (Saxena and Singh, 2011). There is a number of agro industrial residues are available like rice bran, wheat bran, coconut husk, potato peel, sugarcane bagasse etc. Now a day's SSF is mostly used in enzyme production due to low capital investment (the uses of different agro industrial residues), simple technique and good product recovery (Saxena and Singh, 2011). Currently in starch processing industry the chemical hydrolysis of starch is replaced with hydrolysis by amylase. Amylases are mainly used in various industries like liquefaction of starch, textile industry, alcohol, brewing etc. (Forgarty and Kelly, 1979; Gupta et al., 2003).

Production of metabolites by microbes generally depends on the surrounding environments; therefore it is very important to optimize the culture and nutritional conditions of the microbes. The capacity to grow in a given habitat is determined by the organism's ability to utilize the essential nutrients from its surroundings. Organic and inorganic constituents in the medium are exploited for total protoplasmic content, carbohydrate, proteins, amino acids, vitamins, nucleic acids, purine, pyrimidine and other substances that constitute the working apparatus of biological machine. Large polymeric substances are converted to small usable molecules by different hydrolytic action of enzymes which produced by microbes.

There are wide ranges of microbial diversity and each has particular type of nutritional habits. Microbial growth medium basically contains source of carbon, nitrogen, phosphate minerals etc. The supply of oxygen and maintenance of pH of the medium is very important. For the physiological activities and growth of microbes the maintenance of

physical factors that moisture content and temperatures are very important. Generally fungi can survive at low temperature and lower moisture content. Therefore, the optimization of different physical and nutritional parameters is very important for the growth and development of microbes and also for the production of different metabolites like enzyme.

There are two major methods for large scale production of α- amylase: (a) solid state fermentation (SSF) and (b) submerge fermentation (SmF) (Akpan et al., 1999; Soni et al., 2003; Kalairasi and Parvatham, 2013). In SmF (submerge fermentation) for bacterial amylase production mainly synthetic medium is used (Singh et al., 2014). The cost of different synthetic media is very high therefore these media are replaced with low cost agricultural by products to minimize the cost of the medium (Solange and Jose, 2010). The solid substrate provides support and nutrition to the fermentation medium (Hashemi et al., 2012). The solid state fermentation by *Aspergillus oryzae* were mostly reported where *wheat bran* used as the substrate and fermentation environment was at 30 ^{o}C temperature, pH 5.0, where it produces 14967 IU/gds (gram dry substrate) enzyme (Sivaramakrishnan et al., 2007). Suganthi et al. (2011) studied that the alpha amylase production by *Aspergillus niger* in solid state fermentation by using different agro industrial waste.

SSF is preferred over the SmF because of simple process, low capital investment, lower levels of end product inhibition and better product recovery (Okolo et al., 2000; Singhania et al., 2009). Many other substrates are also used in SSF like rice husk, soybean meal, cotton seed meal, rice bran etc. for the production of amylase by *Bacillus* sp (Maryam et al., 2010). The maximum amylase production of 2311.1 U/g by *Bacillus* sp was showed with pH-7, when SSF was carried out at 37 ^{o}C for 48 h using a substrate with 75% moisture content (Saxena and Singh, 2011). The amylase production by *Aspergillus* sp in SSF where wheat bran, rice bran, coconut oil cake, groundnut oil cake, corn cob are used as substrate was studied by Alva et al. (2005) and Pandey et al. (2005). Mathew et al. (2016) also showed that the amylase production by *Aspergillus niger* in solid state fermentation by using cassava peels, jackfruit seeds, coffee husks etc. In presence of soluble starch the amylase activity increases to 1% in respect of glucose and sucrose (Rameshkumar and Sivasudha, 2011). The maximum alpha amylase production was found at 80 h of incubation, 55 ^{o}C temperature, 1:1 substrate moisture ratio, pH 5.0, 10% inoculums level and 0.5g/lit glucose. On the above study glucose was the best carbon

source but the presence of nitrogen source also reduced the production of amylase (Singh et al., 2010).

SSF technique is generally confined to the process involving fungi (Saxena and Singh, 2011). In SSF mainly agro industrial residues are used due to their potential advantage for filamentous fungi which are capable of penetrating the substrate by turgor pressure present at the tip of the mycelium (Maryam et al., 2010). The production of amylase from *Penicillium expansum* in solid state fermentation using waste loquat kernels was studied by Erdal and Taskin (2010). Production of fructo oligosaccharide from agro industrial residues through solid state fermentation by *Aspergillus japonicas* was reported by Solange and Jose, (2010). Production of α- amylase and gluco amylases by *Bacillus* sp and *Aspergillus* sp in solid state fermentation where wheat bran was used as substrate was studied by Soni et al., (2003).

The optimization process has been carried out by one variable at a time (OVAT) and response surface methodology (RSM) etc. The OVAT is time consuming and incapable of detecting the true optimum, due to the interactions among the factors (Pandey et al., 2000) and Response Surface Methodology (RSM) is one of the methods which was used in place of single factor optimization process. In RSM effect of combined factors are studied. It is merging of experimental strategies, statistical inference and mathematical methods (Bernfeld 1955; Baks et al., 2008). Among the optimization parameter temperature, agitation and inoculums size were used for Box- Behken design of response surface methodology (RSM) for α- amylase production by *Aspergillus flavus* (Viswanathan and Surlikar, 2001). Optimization of different parameters and media is very important for the production of large amount of amylase (Annamalai et al., 2011). Juliana et al. (2011) stated that the culture conditions (morphological and metabolic state) are very important factor for wide range of α- amylase production. In case of extracellular enzyme like α- amylase the growth of mycelium is very important (Negi and Banerjee, 2006). There are many physical and chemical factors which affects optimum amylase production such as temperature, pH, incubation period, moisture content, nitrogen source etc. (Elliah et al., 2002).

Temperature

In enzyme production mainly two types of optimum temperatures are required one is for the growth of microbes and another is for the highest production of enzyme. The effect of temperature on amylase is related to the growth of microorganisms (Sivaramakrishnan, 2006; Farouk et al., 2009). The optimum temperature of the medium depends on whether the micro-organism is mesophilic or thermophilic. Sato et al. (2011) asserted that almost all fungi grow between 25-27 ˚C temperature, because they are mesophilic in nature. The increase of temperature also increases the activity reaction kinetics but after a certain temperature the denaturation of protein is occurred which decrease the activity of enzyme. In recent years work has been done on thermostable enzymes obtained from different thermophilic bacteria (Demirijan et al., 2001). Thermostable amylases are isolated from *Bacillus subtilis*, *Bacillus amyloliquifaciens* etc. Thermophilic archaeal amylases are active at high temperature. Thermococcus amylases are active at 80 ^{o}C. Different thermostable enzymes are used in starch industry (Sarikaya et al., 2000). The production of amylase by *Penicillium fellutenum* in solid state fermentation was at 30 ^{o}C reported by Kathiresan and Manivannan (2009). Varalakshmi et al. (2009) stated that *Aspergillus niger* produce optimum amylase at 22 ^{o}C in solid state fermentation. The maximum amylase production by *Aspergillus fumigatus* was at 35 ^{o}C (Singh et al., 2014).

pH

pH also affect the growth of the microorganisms and its effect on enzyme activity is dependent on time and temperature (Elliah et al., 2002). One of the most common acid stable and acid labile molds is *Aspergillus* (Kvesitadze et al., 1978). Some thermostable alkaline enzymes are found from *Bacillus licheniformis* and *Bacillus coagulans* (Medda and Chandra, 1980). *Pyrococcus furiosus* produces alpha amylase at 6.5-7.5 ranges of pH (Haki and Rakshit, 2003). *Thermomyces languginosus* produce 534 U/g amylase by SSF where wheat bran used as substrate at pH 6.0 (Bhargav et al., 2008). *Pyrococcus furiosus* showed optimum activity at pH ranges from 6.5- 7.5 (Haki and Rakshit, 2003). *Saccharomyces cerevisiae* produced maximum enzyme at pH 5.0 (Knox et al., 2004). At

optimum pH of 6.0, 434U/g enzyme is produced by fungi *Penicillium expansam* (Erdal and Taskin, 2010).

Incubation period

Incubation time is a most important parameter in production of amylase. At a certain time the activity of enzyme is highest because at that time the growth of the microbes is high after that the activity decreases due to the depletion of nutrients and release of toxin in the medium (Raul et al., 2014). Most efficient producer of amylase is *Bacillus subtilis* and *Bacillus amyloliquefaciens* which revealed the production of maximum amylase after 72 h of incubation period (Raul et al., 2014). The optimum amylase production by *Bacillus licheniformis* SKB4 was found after 24 h of incubation period (Samanta et al., 2009). Singh et al. (2014) showed that the maximum amylase was produced by *Aspergillus fumigatus* at 6^{th} day of incubation period. The optimum amylase production by *Aspergillus niger* at 72 h of incubation period was reported by Asrat and Girma (2018).

Substrate used for fermentation

There are many different types of substrates have been used for the production of amylase in solid state fermentation like wheat bran, potato peel, banana peel, rice bran, black gram bran, mango kernel etc. Previous report showed that they are very enriched source of substrate for amylase production such as in presence of cassava as substrate maximum amylase (11.0 $kUmg^{-1}$) was produced by *Aspergillus fumigatus* (Pervez et al., 2014) and 1.5U/ml amylase enzyme was produced also by *Bacillus subtilis*, where banana peel used as substrate (Jadhav et al., 2013), in black gram bran *Aspergillus niger* BAN3E produced amylase of 86 U/mg (Ramasamy et al., 2011), coconut oil cake produced 1752 U/gds amylase by *Aspergillus oryzae* (Ramachandran et al., 2004). Pavezzi et al. (2008) showed that *Aspergillus awamori* produce 7U/ml amylase by using soluble starch as substrate.

Moisture content

Moisture is a very important factor in SSF that influence the growth of microorganisms and enzyme production (Pandey et al., 2000). The decreasing and increasing of optimum

moisture content also lower the enzyme production (Elliah et al., 2002). High moisture content reduces the substrate porosity, changes the structure of substrate particles and also decreases the transfer of oxygen. The moisture content required for fungi is less than bacteria for the production of amylase (Leveque et al., 2000). Bacteria require 70-80% initial moisture content for their growth. The production of amylase by *Penicillium janthinellum* required moisture content ranges of 20-80% (Sindhu et al., 2009). The study showed that the maximum enzyme production of 295U/gds was obtained at 60% moisture level but when the moisture content increase to 80% the amount of enzyme production decreased to 220U/gds (Sindhu et al., 2009). In case of *Aspergillus oryzae* the production of amylase (9000U/gds) was maximum at 60% moisture content but the enzyme production decreased when the moisture content increased to 70% (Sivaramakrishnan et al., 2007). The optimum moisture content depends on the substrate used in the fermentation because of its water holding capacity, the substrate with better water holding capacity require lower moisture content. In case of *Bacillus amyloliquifaciens* optimum enzyme production is 3677 U/g at 85% moisture content where wheat bran and ground nut oil cake used as the substrate (Gangadharan et al., 2006). Production of amylase by *Penicillium chrysogenum* with different substrates were studied (like wheat bran, wheat straw, rye straw, corncob leaf) and the production of enzyme was 20, 127,40 and 34U/ ml at 75, 65,65 and 55% moisture content respectively (Balkan and Ertan, 2007).

Inoculum concentrations and particle size

Optimum enzyme production depends on the inoculum concentration and particle size of different solid substrate used (Balkan and Ertan (2007).

Nitrogen source

There are many organic and inorganic nitrogen sources are required in fermentation process for amylase production. The studied organic nitrogen sources were soybean meal, yeast extract, beef extract etc. (Paquet et al., 1991). Addition of nitrogen sources with medium showed higher yield of amylase by *Aspergillus niger* (Pandey et al., 1994) and *Aspergillus fumigatus* (Goto et al., 1998). Yeast extract also increases the production of alpha amylase (Haq et al, 2002). Oshoma et al. (2010) studied that the production of

amylase was effected by nitrogen source using cassava whey as medium by *Aspergillus niger*. They reported a maximum biomass yield of *Aspergillus niger* of 2.75g/l with maximum activity of 643 U/ml, when yeast extract was employed as a nitrogen supplement and the lowest biomass yield of 0.77 g/l and amylase activity of 206 U/ml when sodium nitrate ($NaNO_3$) was used as nitrogen supplement.

Optimization of acidophilic alpha amylase production from *Bacillus cereus* MTCC 10205 in submerged condition using starch medium have been reported by Kumari et al, (2017). Ashwini et al. (2011) also reported that amylase was produced which was 3 fold higher than enzyme produced before optimization. In RSM the amylase activity was 4.6 fold higher than normal basal medium (Zambare, 2011).

In this chapter the degradation pattern of different substrates and the optimization process for maximum amylase production by *Aspergillus niger* RBP7 is discussed.

2.2. Materials and methods

2.2.1. Substrate degradation pattern

2.2.1.1. Uses of different waste materials for amylase production

Different low cost substrates like rice bran, wheat bran, coconut husk, potato peel, sugar cane baggase were exploited for their effect on amylase production. In this study all the substrates were washed, dried and grinded by a mixture grinder with a particle size of around 0.8 mm. After that the substrates were kept in plastic bags with their name, at room temperature for further use. Finally the solid state fermentation (SSF) was carried out in Roux flasks containing 1 g of each low cost agro industrial residues mentioned above used as substrate and moistened with 1 ml of salt solution (%, w/v): [$NaNO_3$ (0.3), $MgSO_4$ (0.05), KCl (0.05), $FeSO_4$ (0.002), K_2HPO_4 (0.1)], of pH 3.0. These flasks were autoclaved and inoculated with 1ml (2×10^2 spore) spore suspension of potent fungal isolate, and subsequently incubated at 27 °C for 72 h in stationary condition. After fermentation the enzyme extraction and assay of amylase by DNS method (Miller, 1959) was carried out. After that, to find out the best substrate the SEM study was carried out before and after fermentation.

2.2.1.2. Scanning electron microscopy (SEM) study

In SEM study, subsequently the fungal mycelia grown on fermented substrate (72 h of fermentation) was fixed in 3.0 % glutaraldehyde and dehydrated with a gradient (10-100 %, v/v) of acetone. The fixed cells were put onto a graphite stub and kept in an auto-sputter coater (POLARON-SC7620) under vacuum for gold coating (Mitchell and Lonsane, 1990). The surface topographical changes of potato peel before and after fermentation was examined using scanning electron microscope (Carl Ziss, Germany).

2.2.2. Estimation of fungal growth

The fungal mycelium of *Aspergillus niger* RBP7 was taken from the actively grown slant and was inoculated into 250 ml Erlenmeyer flask containing 100 ml of Czapek dox broth with slightly modified composition as (in g/l): [$NaNO_3$, (3.0); $MgSO_4$, (0.5); KCl, (0.5); $FeSO_4$, (0.01); KH_2PO_4, (1.0)] and sucrose replaced with starch (1%) and pH was maintained at 3.0. The fungal culture containing flask was incubated at 27 ^oC on shaker (150 rpm). During the fungal growth in liquid medium the mycelium was taken from flask at 0h, 24h, 48h, 72h, 96h, 120h. Then the mycelia were filtered on a Buchner apparatus and their dry weight determined after drying at 60 ^oC for 24h. The growth curve of fungi was constructed from dry cell weights (g/100 ml) versus incubation period.

2.2.3. Inoculums preparation

Spore suspension of *Aspergillus niger* RBP7 was prepared by addition of sterilized distilled water containing Tween 80 (2 drops/ 100 ml) to 3 days-old fungal isolate growing on slants of Czapek dox agar media (containing starch instead of glucose). The final concentration of the spore suspension was adjusted to about 2×10^2 spores/ml and preserved at 4 °C for further use.

2.2.4. Optimization of enzyme production by solid state fermentation

2.2.4.1. One variable at a time (OVAT) approach

Solid state fermentation was carried out in Roux flasks containing 1:1 (g of potato peel as a substrate and moistened with of salt solution (ml) (moistening agent-%, w/v): [$NaNO_3$

(0.3), $MgSO_4$ (0.05), KCl (0.05), $FeSO_4$ (0.002), K_2HPO_4 (0.1)], of pH 3.0. These flasks were autoclaved and inoculated with 1ml (2×10^2 spore) of RBP7 fungal isolate and subsequently incubated at 27 °C for 72 h.

In this study potato peel was used as an effective substrate for amylase production. The effect of pH on enzyme production was checked by preparing the salt solution at variable pH (2.0-5.0). The effect of temperature on enzyme production was analyzed by incubation at different temperatures (25°-50°C). The amount of substrate material was varied (0.25 g-1.75 g) to check its effect on enzyme production. Further inoculums concentration (0.25 ml–2.0 ml/ g) and the incubation period (0-96 h) of enzyme production were studied. The effect of initial moisture contents (0.25 ml-2.0 ml/ 1g dry substrate) were also experimented and optimized for better enzyme production. In order to determine the effect of nitrogen source on amylase production several inorganic and organic nitrogenous compounds ($NaNO_3$, NH_4NO_3, KNO_3, peptone, casein, yeast extract) were supplemented separately at 0.3% level to liquid medium and checked for enzyme production. Various percentage of inorganic component of Czapek dox medium were added [$MgSO_4$(0.13% - 0.17%, w/v),KCl(0.13% - 0.17%, w/v), $FeSO_4$ (0.004% - 0.008%, w/v), K_2HPO_4 (0.1% - 0.5%, w/v)] into the SSF medium for maximum production of α-amylase.

2.2.4.2. Statistical optimization using response surface methodology (RSM)

Box–Behnken factorial design was employed in the optimization of culture conditions for amylase production. The four more influencing factors screened during OVAT optimization were employed in RSM study. A total 27 runs comprising four factors at their three level (-1, 0, + 1) were designed and carried out experimentally.

The response Y was amylase activity [U/gram dry substrate (gds)]. The equations of the regression model were incorporated where xi and xj are the input variables, which influence the response variable Y, β0 the offset term, βi the ith linear coefficient, βii the quadratic coefficient, and βj is the ijth interaction coefficient. The response surfaces of the variables inside the experimental domain were analyzed using Design Expert software

(Version 8.0, Stat-Ease Inc., USA). A polynomial quadratic equation was adopted to evaluate the contribution of each independent variable in this process

$Y = \beta_0 + \sum \beta_i x_i + \sum \beta ii Xi^2 + \sum \beta ij Xi Xj$

2.2.4.3. Enzyme extraction

After fermentation sterile distilled water was added to each flask (substrate : distilled water = 1:5 w/v) and vigorously agitated in shaker for 30 min at 100 rpm. The mixture was filtered through cheese cloth and centrifuged at 8,000 rpm for 10 min. The supernatant was considered as the crude enzyme preparation and used for subsequent study.

2.2.4.4. Assay for α-amylase activity

The activity of α-amylase in the fermented broth was determined by following method of Miller (1959). Briefly, 0.5 ml of crude enzyme was incubated with 1 ml of soluble starch (1%, w/v) prepared in acetate buffer (0.2 M, pH 3.0) at 37 °C for 60 min. The reaction was terminated by the addition of 1 ml of 3, 5- dinitrosalicylic acid (DNS) reagent. The released reducing sugars were estimated colorimetrically at 540 nm. One unit of amylase activity was defined as the amount of enzyme releasing 1 μg of reducing sugars (glucose equivalents) per minute under standard assay condition.

2.2.4.5. Protein determination

To measure the protein content of the enzyme extract Lowry method (Lowry et al., 1951) was used, where bovine serum albumin (BSA) was used as the standard. At first, 4.5 ml of the reagent I (48 ml of 2% Na_2CO_3 in 0.1 N NaOH, 1 ml of 1% NaKtartarate in H_2O and 1 ml of 0.5% $CuSO_4.5H_2O$ in H_2O) was added in a test tube and incubated for 10 min. After incubation 0.5 ml of reagent II (1 part Folin-Phenol [2N]: 1 part water) was added in the same test tube and mixed the two reagents. After that 1ml of crude enzyme was added to the reagent mixture and incubated for 30 min. Finally the presence of protein in the extracted crude enzyme was measured spectrophotometrically at 660 nm.

2.2.4.6. Production of acidophilus amylase in large scale

The large scale acidophilus amylase (the amylase enzyme was stable also in acidic pH or low pH) production in SSF was performed by using the static trays (1 ft by 2 inch) Each of the trays was filled with 250 g of potato peel and moistened with 250 ml of salt solution (% w/v): [$NaNO_3$ (0.3), $MgSO_4$ (0.05), KCl (0.05), $FeSO_4$ (0.002), K_2HPO_4 (0.1)], of pH 3.0 and inoculated with 175 ml of spore suspension (2×10^2 spores/ ml) and incubated at 40 ^oC for 72 h in a closed humidity chamber (REMI Environmental Chamber, CHM6S, India).

2.2.5. Optimization of Submerged fermentation for acidophilic amylase production

2.2.5.1. OVAT methodology

Acidophilic amylase production in submerged fermentation by *Aspergillus niger*RBP7 was carried out in Erlenmeyer flasks (250 ml) contained 100 ml fresh liquid media (same composition used during primary screening and media also supplemented with potato peel) for 120 h at 27 °C on a rotary shaker (120 rpm). The cultural conditions were optimized by studying one variable at a time (OVAT). The physical parameters like fermentation time, pH, temperature, substrate concentration, inoculums concentration were also optimized with their specific integrity by classical optimization technique i.e. OVAT approach. After the production of amylase the activity of enzyme was measured by DNS method of Miller (1959).

2.2.5.2. Statistical approach by using Response Surface Methodology

Box Behnken Design (PBD) was employed for screening the most significant parameters affecting the concomitant production of acidophilic amylase by *Aspergillus niger* RBP7. The predictor variable was examined at high and low levels, each of which are indicated by (+) and (-) symbol. Four factors namely optimum initial pH, optimum fermentation temperature (°C), substrate concentration, inoculums concentration were screened in 27 experimental runs and the responses in terms of amylase activity. After preliminary screening, a Box-Behnken design (BBD) was employed for further optimization of the

effective level of four most influencing factors for production of acidophilic amylase. Three equidistant levels [low (-1), middle (0) and high (+1)] of each factor encompassing of 27 experiments (in triplicate) were conducted.

Statistical analysis was done by Design Expert 8.0. A model was generated by the regression analysis and its efficiency was tested by ANOVA and Fisher's F-test. Mutual interactions of the variables were represented by three dimensional response surface plots.

2.2.5.3. Comparative study of acidophilic amylase from *Aspergillus niger* RBP7 and standard strain *Aspergillus niger* MTCC 281

In this study, a comparison has been made with the production of acidophilic amylase from *Aspergillus niger* RBP7 in submerged fermentation (in optimized condition) and *Aspergillus niger* MTCC 281 in its standard medium Czapek yeast extract 100 ml of liquid medium (LM) (%, w/v): ml [($NaNO_3$ (0.3), $MgSO_4$ (5.0), KCl (0.050), $FeSO_4$ (0.001), K_2HPO_4 (1.0), Yeast extract (5.0), sucrose (30.0)] and pH was adjusted at 3.0. These flasks were autoclaved and inoculated with 1ml (2×10^2 spore) of *Aspergillus niger* MTCC 281, and subsequently incubated at 30 °C for 7 days according to MTCC standard protocol.

2.3. Results and discussion

2.3.1. Production of amylase in solid state fermentation by using different substrates

The amylase was produced by RBP7 in solid state fermentation where rice bran, wheat bran, coconut husk, potato peel, sugarcane bagasse used as substrate and the fermentation was carried out at 27 °C for 72 h. The pH of the medium was maintained at 3.0. Among the tested substrates potato peel, wheat bran and rice bran, showed significant enzyme production by176.3 U/gds, 150.65 U/gds, 140.5 U/gds respectively. The rate of enzyme production was increased by following order potato peel > wheat bran > rice bran > sugarcane bagasse > coconut husk (Table 2.1). Several researchers also used various substrates for production of fungal amylase through SSF. Sugarcane bagasse was used as substrate for amylase production from *Aspergillus niger* and produced 109.12 U/gds enzyme (Roses and Guerra, 2009). The strain *Penicillium chrysogenum* produced

759U/gds amylase when grew in wheat bran as substrate (Ertan et al., 2006). Kalairasi and Parvatham (2015) reported that *Aspergillus awamori* produced 20.68 U/gds amylase in rice bran as substrate. *Aspergillus niger* was also tested in potato peel as substrate and produced 1262.27 U/gds amylase (Mahmood et al., 2016).

Table 2.1 Production of amylase by RBP7 in different low cost starchy materials

Substrates used for enzyme production	**Enzyme production (U/gds)**
Rice bran	140.5
Wheat bran	150.65
Coconut husk	120.5
Potato peel	176.3
Sugar cane bagasse	137.8

Plant-based solid substrate is suitable for microbial enzyme production is particularly due to the availability of inducer molecule and less amount of anti-nutrients (Dash et al., 2015). Potato peel as a suitable substrate for α- amylase production by *Bacillus* strain was reported in earlier studies by Shukla and Kar (2005). Potato peel is a better supporting substrate for fungal growth probably due to the presence of various available nutrients like proteins 18 %, carbohydrates 55.0 % (starch 25.0 % and 30 % non-starch polysaccharides like xylans, cellulose, and glucan), fats 1.0 %, ash 6.0 %, Ca 0.12 %, Mg 0.23 %, P 0.57 %, K 0.42 %, Na 0.06 %, and various amino acids and vitamins (Mitchell and Lonsane, 1990). Large quantities of essential nutrients including inducer starch and increased surface area of potato peel may provide optimum support for α- amylase production from *Aspergillus niger* RBP7 in respect to other tested substrates. Therefore, in present study the SSF fermentation is carried out by using potato peel as substrate. Mahmood et al. (2016) stated that potato peels have good porosity, suitable particle size and consistency required for anchorage and enzyme excretion. Its texture remains loose even in moist condition, thereby providing a large surface area by holding water (Laddish et al., 1983).

2.3.2. Analysis of substrate degradation pattern

2.2.2.1. SEM study

The internal structure of potato peel was studied by scanning electron microscope before and after α- amylase production by *Aspergillus niger* RBP7. The control potato peel showed a regular alignment of cells without any damage was observed under scanning electron microscopy (Fig. 2.1 a, b). After fermentation the surface of potato peels become rough, slacken and dispersed (Fig. 2.1 c, d). This might be due to the effect of *Aspergillus niger* RBP7 fermentation and their enzymes (Mukherjee et al., 2017). Our result is in ascendance with the work of Mu et al. (2015) where α- amylase exhibhited similar hydrolytic effect on potato peel.

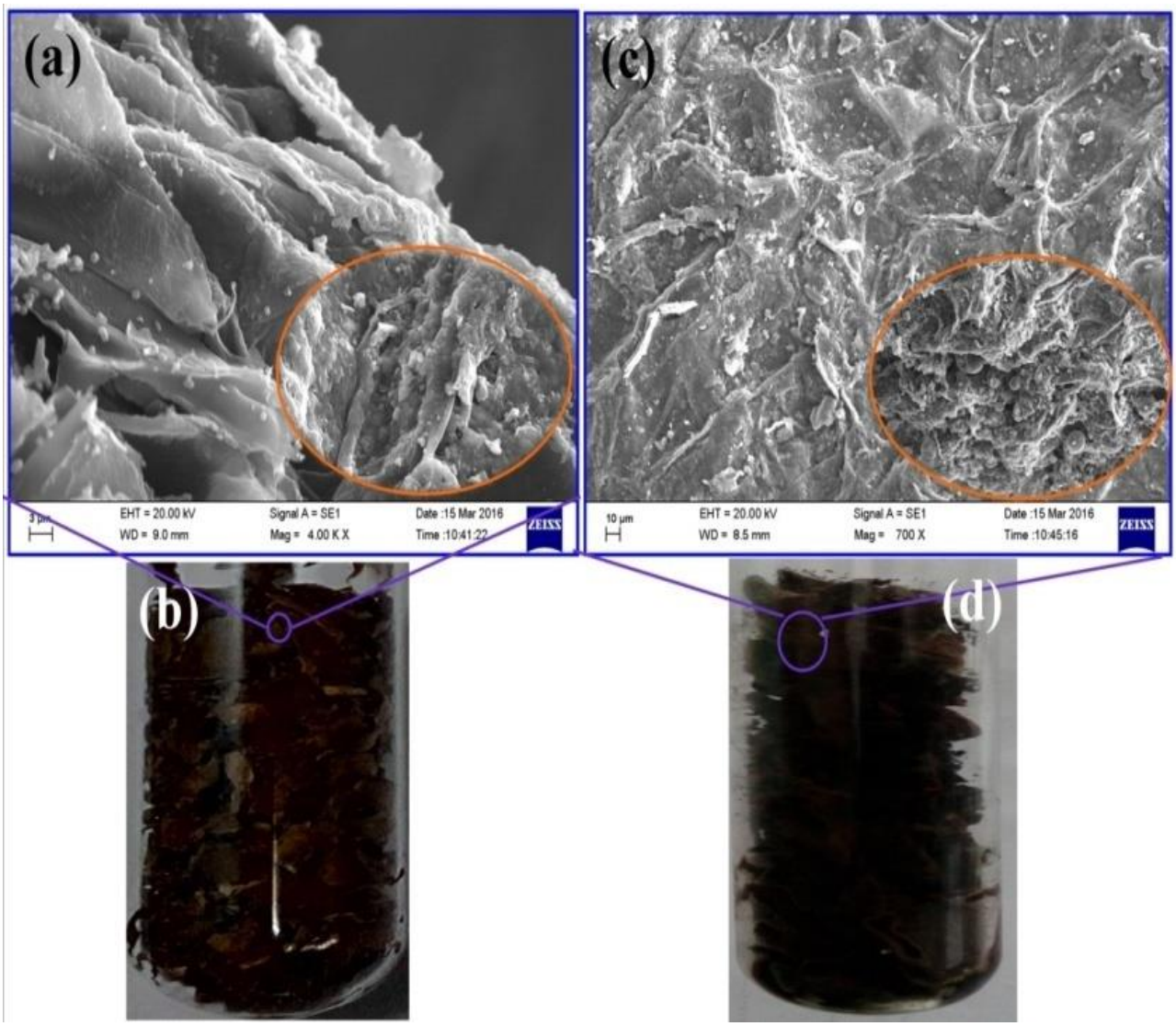

Fig 2.1. Phenotypic appearance (b, d) of potato peels and details surface structural changes of potato peel was determined under SEM. a) Control potato peels and c) potato peels after the fermentation. Orange circle indicate that the closer view of the potato peels surface structure.

2.3.3. Growth curve of fungi

The growth curve showed a log phase from 48h to 72 h, stationary phase from 72 h to 96 h. After that period, the mycellial mass was decreased in 130 h because of nutrient depletion after 96h of incubation (Fig. 2.2). Melgar et al, (2013) stated that *Aspergillus sydowii* showed the log phase from 48 to 144 h and decline phase after 192 h of incubation.

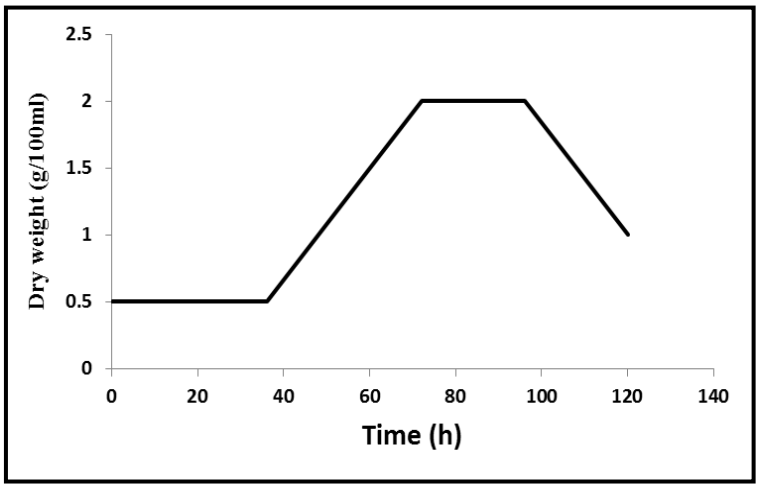

Fig 2.2. Growth curve of *Aspergillus niger* RBP7

2.3.4. Optimization of fermentation parameters of solid state fermentation

2.3.4.1. OVAT methodology

The amount of substrate material, a major factor for the production of amylase in SSF by *Aspergillus niger* RBP7 was studied by varying the amount of potato peel (0.25- 2.25 g/10ml). The optimum enzyme production was 176.30 U/gds (Fig. 2.3) at 1.0 (g/10ml) of potato peel. The amylase production was also increased with the increase of potato peel amount. However, the amylase production gradually decreased above 1.0 % (g/10ml) of potato peel. Higher amount of substrate increases compaction and viscosity of the medium, leads to interfere with oxygen supply. Lack of oxygen supply lowers the energy production in aerobic organisms and decreases anabolic reactions as well as amylase synthesis (Samanta

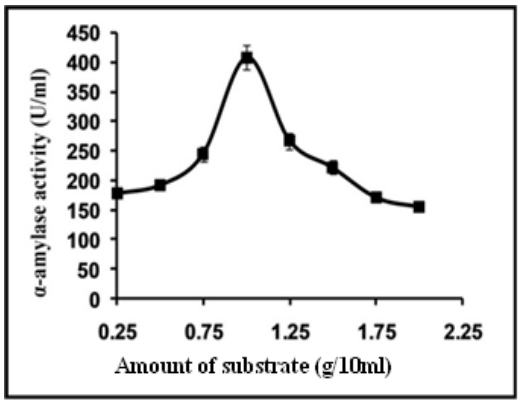

Fig 2.3. Effect of the amount of substrate material (potato peel) on acidophilus amylase production of *Aspergillus niger* RBP7. The amount of substrate of the medium was 1.0 (g/10ml) in SSF

et al., 2014). Previously 9065 U/gds amylase was produced from *Aspergillus oryzae* by using 5 g of wheat bran (Sivaramakrishnan et al., 2007).

The pH of the medium is one of the important factors that determined the growth and enzyme secretion by microorganisms (Kalaiarasi and Ramasamy, 2013). The activity and three dimensional structure of α- amylase from *Aspergillus niger* RPB7 was found to be significantly high at pH 3.0 (274.45 U/gds) (Fig. 2.4). In solid state fermentation the salt solution was mixed with potato peel and after mixing the pH of the potato peel containing salt solution were around 3.0, which help the growth of the fungus and subsequent amylase production. Generally fungi prefer slightly acidic conditions and the pH affects in enzyme productivity because it influences the solubility of substrates in medium and also maintain the ionization process of the substrate which is necessary for the mold's growth. Previous report indicated that there is no symmetry between the optimal pH for microorganism growth and the optimal pH for enzyme activity (Cheesbrough, 2005). Nguyen et al., (2000) found that the optimal pH for α-amylase production by *Thermomyces lanuginosus* ATCC 34626 was 4.9 and produced 125 U/ml amylase. Previous report stated that 13U/mg amylase was produced at pH 5.5 by *Aspergillus niger* BAN 3E (Ramasamy et al., 2011). Several authors (Irfan et al., 2012; Manivannan et al., 2015) reported that

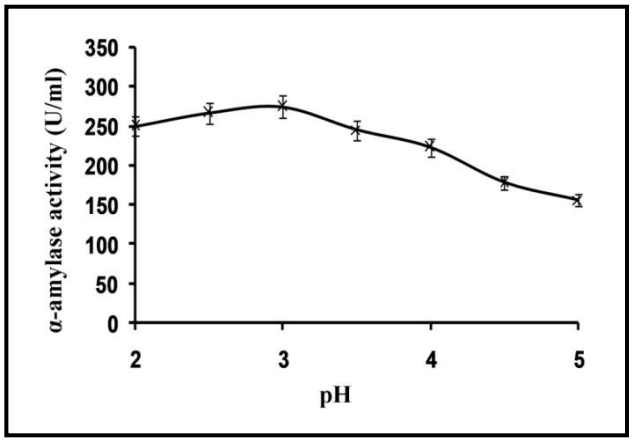

Fig 2.4. Effect of incubation pH on acidophilus amylase production of *Aspergillus niger* RBP7. The pH of the medium adjusted at 3.0 in SSF

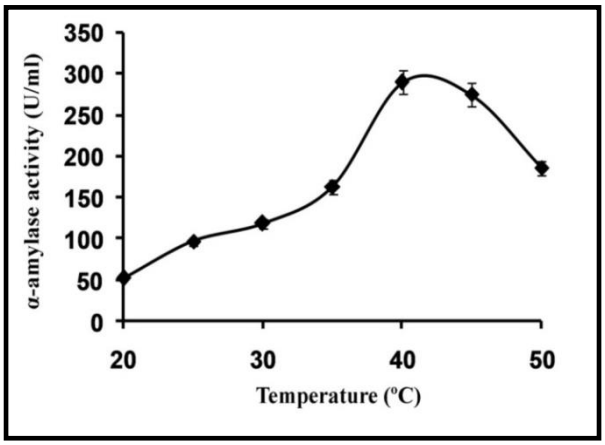

Fig 2.5. Effect of incubation temperature on acidophilus amylase production of *Aspergillus niger* RBP7. The temperature of the medium adjusted at 40 °C in SSF

Aspergillus niger ML-17, *Rhizopusoligosporus* ML-10 and *Aspergillus flavus* produced maximum amylase at pH 6.0.

Alpha amylase production by *Aspergillus niger* RBP7 increased significantly and reached maximum (289.50 U/gds) at 40 °C. Above 40 °C of enzyme production declined, which might be due to adverse effect of higher temperatures (Fig. 2.5). Higher temperature increases the membrane fluidity and also enhances the chance of disruption of membrane beside this high temperature disrupt the three dimensional structure of protein. These thermal mediated changes are exerting detrimental effect on microbial cell as well as enzyme production and secretion. Previous study showed that maximum amylase was produced by *Aspergillus fumigatus* (337.4U/ml)at 35 °C (Singh et al., 2014), *Aspergillus oryzae* (4.137U/ml) at 45 °C (Shah et al., 2014) and another strain of *Aspergillus oryzae* (2.76 IU) at 30°C (Puri et al, 2013).

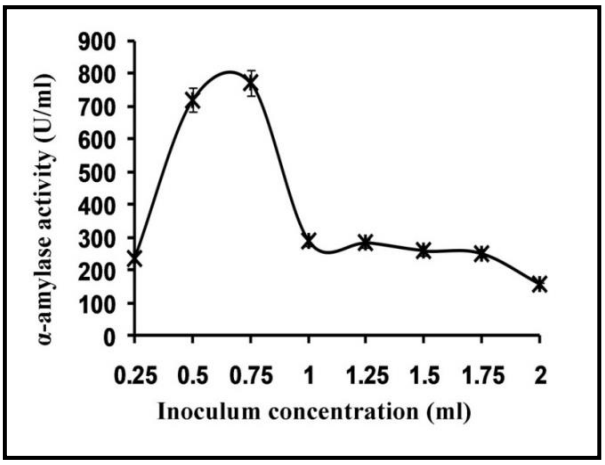

Fig 2.6. Effect of inoculums concentration on acidophilus amylase production by *Aspergillus niger* RBP7. The inoculums concentration was

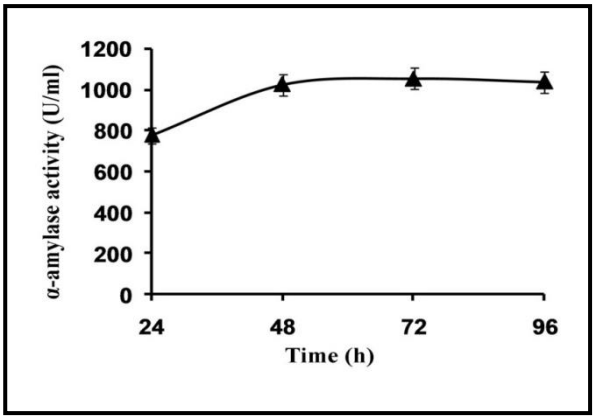

Fig 2.7. Effect of incubation period on acidophilus amylase production by *Aspergillus niger* RBP7. The incubation period was adjusted to 72 h in SSF

The fermentation profile of an organism is usually affected by the initial inoculums concentration. There was a gradual increase in the α-amylase production with increase in inoculums size and reached at 771.45 U/gds at 0.75 ml/g substrate (Fig. 2.6) (1ml= 2×10^2 conidia).

Further increase in inoculums level, resulting in increased competition among fungal cells for carbon source and nutrition, which might lead to exhaustion of nutrients. On the other hand lower inoculums levels contains lesser number of cells in the production medium resulting longer time required for fermentation and enzyme production (Nguyen et

al., 2000). Previously Kalaiarasi and Parvatham (2013) claimed 10% inoculums were reported as optimum for α-amylase production by *Bacillus cereus.* Zambare (2010) and Bhardwaj et al. (2012) found that at 5-10% inoculums concentration produced maximum amylase (1672-1693 U/gds) and (3.873 U/gds) by *Aspergillus oryzae* and *Aspergillus flavus* respectively.

A low level of α-amylase obtained in the early stages of SSF became steadily reached a maxima [1053.35 U/gds] at 72 h (Fig. 2.7). A prolonged incubation

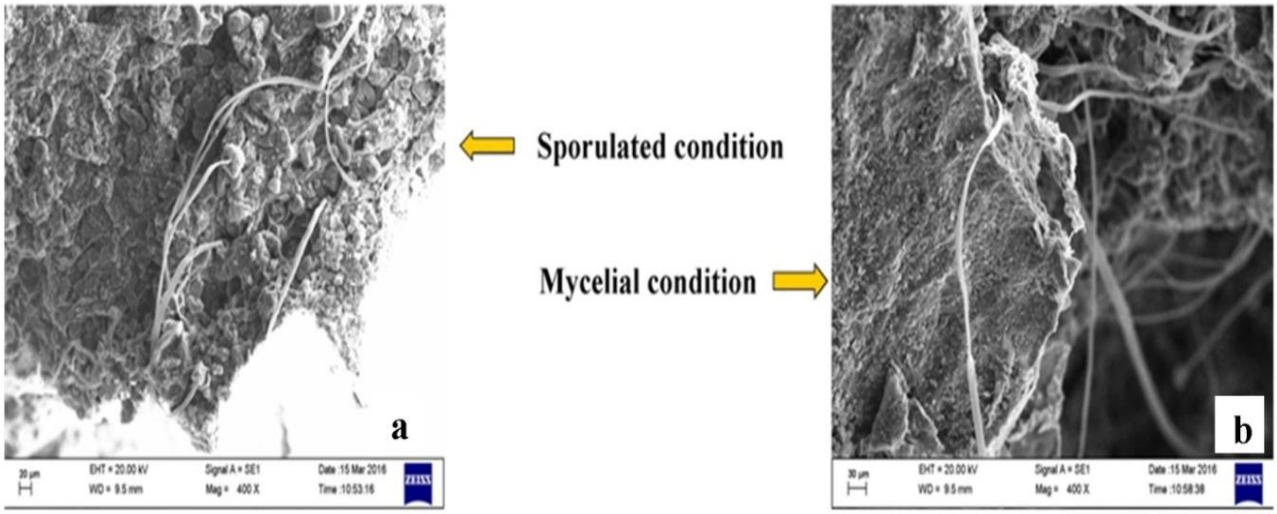

Fig 2.8. Scanning electron microscopic observation of (a) sporulation growth and (b) mycellial during SSF

resulted into sharp decline of enzyme level in fermented mass (Fig. 2.7).This may happen due to the autolysis of fungal hyphae and subsequent accumulation of organic acid.

The SEM studies showed healthy growth of fungi on peels as a thin layer of mycellial aggregates (Fig. 2.8 b). At 72 h of growth, fungi remained in mycelial state (Fig. 2.8 b) and after that it became sporulated (Fig. 2.8 a). This indicated that mycelia state of *Aspergillus niger*RBP7 is more active for induction as well as for secretion of acidophilus amylase. The optimum amylase production by *Aspergillus niger*at 72 h and 96 h of incubation period was reported by Abu et al. (2005) and Ahmed et al. (2014) respectively. Karri et al. (2014) reported that the highest amylase was produced by *Aspergillus* strain at 72 h of incubation period.

Moisture level is a critical parameter for optimizing SSF. The enzyme production was increased to 1060.75 U/gds in the presence of 1.25 ml/g dry substrate in fermentation

environment. Similar observations were noticed in the literature (Alva et al., 2005; Deng et al., 2005) during SSF studies for amylase production. The result revealed that the moisture level has significantly influence the enzyme production by the strain during SSF. The moisture content of SSF regulates metabolic efficiency of microbes that also determined the growth of fungal mycelia into the surface of the substrate with low moisture content (Laddish et al., 1983). Free excess liquid in the SSF system increases the content of the unabsorbed fluid which acts as additional diffusion barrier together with solid nature of the substrate. These effects might lead to a decrease in growth and enzyme production (Alva et al., 2005).

Among the different organic and inorganic nitrogen supplementation, yeast extract at 0.3 % (w/v) promoted high α-amylase biosynthesis (1090.40 U/gds) by SSF. Previous study reported that yeast extract served as an ideal organic nitrogen source for α-amylase production (Bedan et al., 2014; Singh et al., 2014). The addition of yeast extract in a low level (<5 g/l) showed a significant stimulatory effect on enzyme production by *Bacillus licheniformis* (Fattah et al., 2013). Oshoma et al. (2010) showed that 2.0 g (w/v) of yeast extract produced maximum amylase (643U/ml). Pandey et al. (2005) stated that the different organic or inorganic nitrogen sources have immense effect on enzyme production by SSF. Yeast extract not only served as a source of nitrogen but also considered as a rich source of vitamins and many growth factors (Guerra and Pastrana, 2002). Previous research showed that casein, peptone were most important nitrogen sources for the production of amylases by *Aspergillus oryzae* (Pederson and Neilson, 2000).

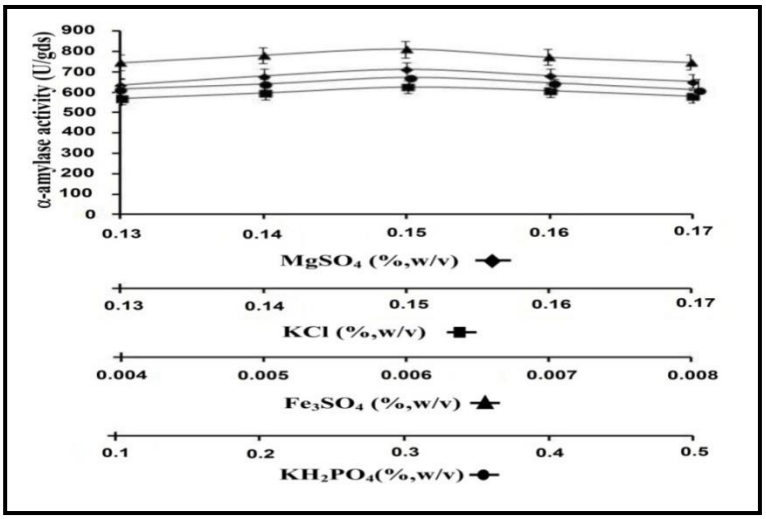

Fig 2.9. Production of acidophilus α-amylase by SSF in the presence of different concentration of inorganic substances by *Aspergillus niger* RBP7

Among the different salt concentration maximun 769.5 U/gds amylase was produced in presence of following salt concentration:
[KCl at 0.15% (w/v), 557.8 U/gds; KH_2PO_4 at 0.3% (w/v), 626.6 U/gds; $MgSO_4$ at 0.15% (w/v), 666.5 U/gds; Fe_3SO_4 at 0.006% (w/v)] (Fig. 2.9). Previously Kunamneni et al. (2005) stated that in the presence of $NaNO_3$, NH_4NO_3 and salt of different minerals (Fe, Mg, Ca, Zn) used for maximum enzyme production (388U/g) by *Thermomyces lanuginosus*. The protein content of the acidophilus amylase after OVAT was 32.47 mg/ml measured by Lowry method.

2.3.4.2. RSM study

Statistical optimization by experimental design offers the opportunity to find out the optimal levels of process variables. The amylase production in SSF was optimized by RSM methodology (Box- Behnken design) where 27 runs combined with various four factors namely initial pH, fermentation temperature (30°C), amount of potato peel (g/10 ml) and inoculums concentration (ml/g) were carried out. For each run, the experimental responses along with the speculate responses were calculated from the regression equation (Eq. 1) and was shown in (Table 2.3). The data were analyzed by multiple regression analysis and the regression coefficients were determined (Table 2.2). A second-order polynomial equation (Eq. 2) was achieved to signify the amylase production as a function of the independent variables tested.

$$Y_{\alpha\text{-amylase}}=378.10+51.56\times A-87.16\times B+127.18\times C+110.21\times D-85.27\times AB+77.93\times AC-101.92\times AD+225.63\times BC+29.92\times BD+148.44\times CD-65.42\times A^2-47.57\times B^2+218.56\times C^2+83.16\times D^2 \quad \text{.....(2)}$$

Where, Y is the speculate responses, and A, B, C, and D are used as values of initial pH, fermentation temperature (°C), amount of potato peel (g/10 ml) and inoculums concentration (ml/g) respectively. Adequacy of the model was tested by the Fisher's statistical test for the ANOVA using Design Expert software, and the results are shown in Table 2.3. ANOVA of the quadratic regression model suggesting that, the model is significant with a computed F value of 29.85 and P>F value is lower than 0.05. A lower value for the coefficient of variation suggested higher reliable experiment, and in this case,

the result documented a higher reliability of the trials. The R^2 value obtained was 0.9873, indicating that 98.73 % of the sample variation is attributing to the factors and the left can occur due to chance. Table 2.2 also gives the P values of each of the parameters and their quadratic and interaction terms. The significant individual variables can be tested from their P values, the more significant terms having a lower P value. An adequate precision of 34.397 indicated low signal to-noise ratio. Response surface curves were plotted to understand the interaction effects of variables and for identifying the optimal levels of each parameter for attaining maximal α- amylase yield. The response surfaces obtained for the interaction effects of tested variables, where two factors were varied and others were kept at their central point (Fig. 2.10). The shapes of contour plots indicate the nature and extent of the interactions. The significant interactions between tested variables were observed from the Response surface and Contour plot. It is clearly observed from the response surface and contour plot that there are significant interactions between the tested variables. From the analysis, it was predicted that the maximum α-amylase yield of 1112.25 U/gds was obtained at the following fermentation condition: amount of potato peel (1.25 g/10 ml), incubation temperature (44.46 °C), initial pH (2.74) and inoculums concentration (0.99 ml/g). Practical validation of the RSM model was carried out by performing fermentation experiment by providing the model suggested conditions. In OVAT maximum amylase production (1090.40 U/gds) was observed at pH 3.0, temperature 40 ^{o}C, amount of substrate 1% (w/v), inoculums concentration 0.75 ml/g substrate which was in good assessment with the predicted results (1112.25 U/gds) of RSM. The enzyme production was increased in RSM than OVAT due to the effect of interactions between four different variables. Kwatia et al. (2017) showed that amylase production by *Aspergillus niger* was increased from 29.66 U/ml (actual value) to 30.95 U/ml (predicted value) by the interaction between temperature and pH. The production of amylase by *Penicillium notatum* NCIM 923 was increased from 2810.33 U/g (actual value) to 2819 U/g (predicted value) by interaction among the following parameters: amount of substrate, initial moisture, fermentation time, temperature and inoculums size. Mustafa et al. (2016) showed that predicted value (1.022U/ml) is almost similar with actual value (1.055U/ml) of amylase where pH, temperature, incubation period and moisture content were used as parameters for RSM. Therefore, in large scale production this process can be used.

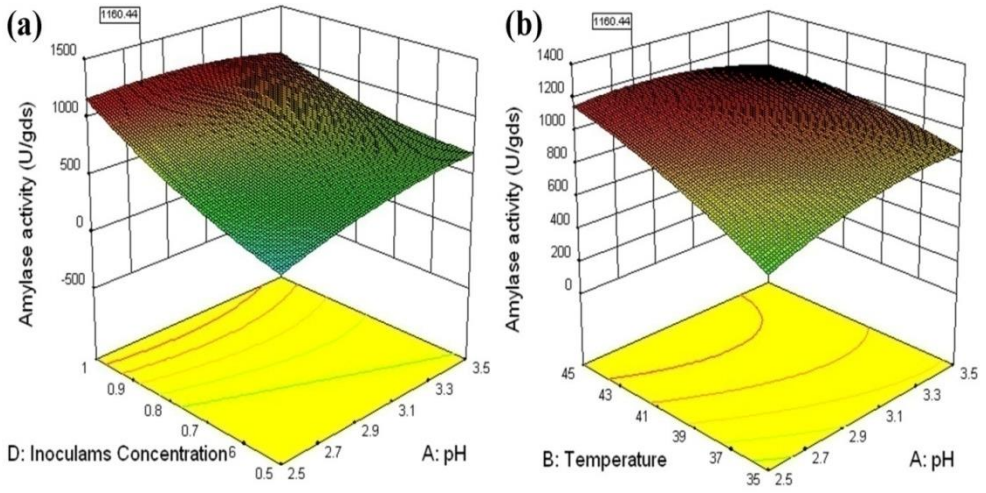

Fig 2.10. Response surface and three-dimensional contour plot (SSF) of the combined effects of a) inoculums concentration (conidia/gds) and medium pH, b) temperature (°C) and medium pH on α-amylase production by *Aspergillus niger* RBP7 in SSF

Table 2.2. Experimental design used in RSM studies by using four independent variables each at three levels showing observed predicted values of acidophilus α- amylase production by *Aspergillus niger* RBP7

Run	Factor 1 A:pH	Factor 2 B:Temparature	Factor 3 C:Substrate	Factor 4 D:inoculums	Response 1 Amylase activity U/gds	Predicted value
1	3.5	35	1	0.75	526.4	489.10
2	3	40	0.75	1	531.8	514.41
3	3.5	40	1.25	0.75	793.4	787.90
4	2.5	40	1.25	0.75	480.7	528.93
5	3	45	1	1	460.8	466.66
6	3.5	45	1	0.75	148.3	144.23
7	3	35	0.75	0.75	711.8	734.69
8	3	35	1.25	0.75	524.6	537.80
9	3	40	0.75	0.5	570.1	590.86
10	3	40	1	0.75	378.1	378.10
11	3	40	1.25	1	1112.25	1065.65
12	3.5	40	1	0.5	399	439.11
13	3	40	1.25	0.5	556.8	548.35
14	3.5	40	1	1	404.5	455.68
15	3	40	1	0.75	378.1	378.10
16	3	40	1	0.75	378.1	378.10
17	3.5	40	0.75	0.75	422.1	377.69
18	2.5	40	1	1	574.5	556.41
19	2.5	40	0.75	0.75	421.1	430.42

20	3	35	1	1	556.1	581.13
21	3	45	1	0.5	207.6	186.39
22	2.5	45	1	0.75	200.2	211.66
23	3	45	0.75	0.75	100.3	109.12
24	3	45	1.25	0.75	815.6	814.73
25	2.5	35	1	0.75	237.2	215.43
26	2.5	40	1	0.5	161.3	132.14
27	3	35	1	0.5	422.6	420.56

Table 2.3. Analysis of varience (ANOVA) for acidophilic α- amylase production by *Aspergillus niger* RBP7 in SSF

Source	Sum of squares	Df	Mean square	F value	p-value Prob>F	
Model	1.296E+006	14	92536.61	66.48	<0.0001	Significant
A-pH	31899.14	1	31899.14	22.92	0.0004	
B-temperature	91158.90	1	91158.90	65.49	<0.0001	
C-Substrate	1.941E+005	1	1.941E+005	139.44	<0.0001	
D-Inoculum	1.458E+005	1	1.458E+005	104.72	<0.0001	
AB	29087.30	1	29087.30	20.90	0.0006	
AC	24289.22	1	24289.22	17.45	0.0013	
AD	41554.82	1	41554.82	29.85	0.0001	
BC	2.036E+005	1	2.036E+005	146.29	<0.0001	
BD	3582.02	1	3582.02	2.57	0.1347	
CD	88134.77	1	88134.77	63.32	<0.0001	
A2	22827.51	1	22827.51	16.40	0.0016	
B2	12070.31	1	12070.31	8.67	0.0123	
C2	2.548E+005	1	2,548E+005	183.03	<0.0001	
D2	36881.64	1	36881.64	26.50	0.0002	
Residual	16703.26	12	1391.94			
Lack of FR	16703.26	10	1670.33			
Pure Error	0.000	2	0.000			
Cor Total	1.312E+006	26				

2.3.4.3. Pilot scale experiment for production of amylase by solid state fermentation

In pilot scale experiment for the production of acidophilus amylase by *Aspergillus niger* RBP7 in the optimized solid state media was carried out in static trays. In this experiment potato peel is also used as substrates for amylase production. In this system *Aspergillus niger* RBP7 produced 1105.6 U/gds acidophilus amylase which was almost similar with the result of OVAT (1090.40 U/gds). The amylase production in large scale fermentation

was reported by Sindhu et al. (2009) and Hassan et al. (2015) and the organisms were *Penicillium* sp. and *Bacillus* sp respectively where wheat bran and rice straw were used as substrate.

2.3.5. Optimization of submerged fermentation

2.3.5.1. OVAT methodology

Maximum production of acidophilic amylase (115.58 U/ml) by *Aspergillus niger* RBP7 in SmF was achieved after 120 h (Fig. 2.11) in the synthetic media containing potato peel 4g/100 ml (w/v) (Fig. 2.12), inoculums concentration 1.25 ml (v/v) (Fig. 2.13), temperature 35 ºC (Fig. 2.14) and of pH 4.0 (Fig. 2.15). Alpha amylase production by *Aspergillus* species in submerged fermentation was studied by many authors (Sani et al., 1992; Gomes et al., 2005; Gupta et al., 2008; Morya and Yadav, 2009). The maximum amylase production by submerged fermentation at optimized condition was studied by Singh et al. (2012) and Esfahani et al. (2008). The incubation period is a major factor for production of amylase. Kathiresan and Manivannan (2006), Mamma et al. (2008) stated that maximum amylase was produced at 96 h of incubation under submerged fermentation by *Penicillium fellutanum* as compare with the present result (120 h). The pH is an essential factor that determined the growth and overall metabolism of microorganisms. The structure of protein depends on the hydrogen ion concentration of the environment. The optimum amylase production by *Aspergillus niger* and *Rhizopus stolonifer* at pH 6.0 was studied by Saleem and Ebrahim (2014) which is slight higher than the optimum pH (4.0) of present isolate for amylase production. The highest amylase production by *Aspergillus niger* UO 1 at pH 5.0, was studied by Hernadnez et al., (2006). The maximum α-amylase production (6.21 U/ml) by *Aspergillus fumigatus* (inoculums size 6×10^6) was studied by Ahmed et al., (2015). The temperature of medium regulates the enzyme production. Above the optimum temperature the denaturation of protein is occurred therefore the enzyme production gradually

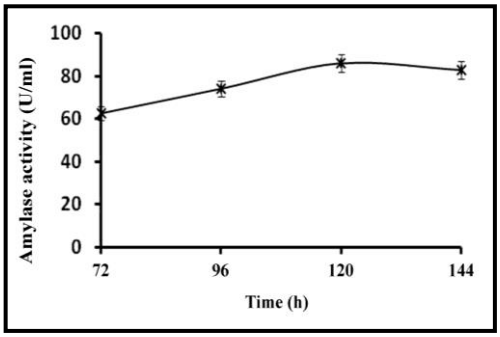

Fig.2.11. Effect of incubation period on acidophilus amylase production by *Aspergillus niger* RBP7. The incubation period was adjusted to 120 h inSmF

decreases. The optimum temperature for amylase production was found at 30 ºC (Goyal et al., 2005; Kathiresan & Manivannan, 2006; Alva et al., 2007) which is slightly lower than the present isolate (temperature optima 35ºC). The substrate controls the oxygen supply, anchorage of fungal mycelia and enzyme production. Previous study attested that when 1% potato starch encourage maximum amylase production in submerged fermentation by *Chrysosporium asperatum* (Sanghvi et al., 2011).

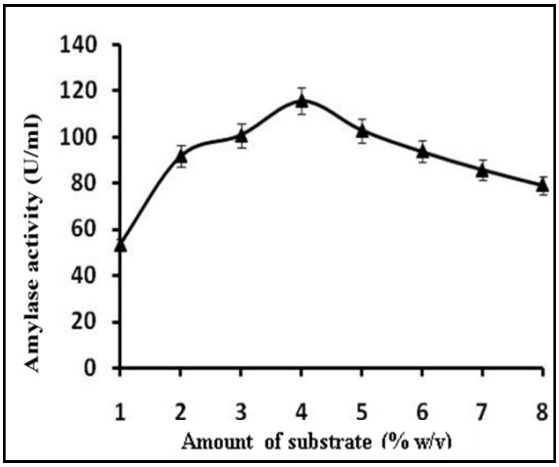

Fig 2.12. Effect of amount of substrate material on acidophilus amylase production in SmF by *Aspergillus niger* RBP7. The amount of substrate was adjusted at 4g (%w/v)

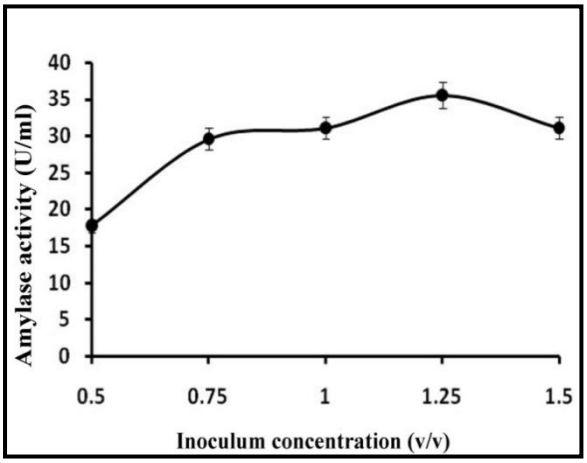

Fig 2.13. Effect of inoculums concentration on acidophilus amylase production in SmF by *Aspergillus niger* RBP7. The inoculums concentration was adjusted at 1.25 ml (v/v)

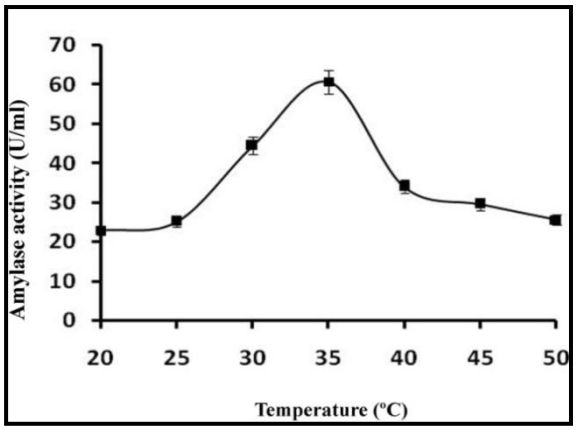

Fig 2.14. Effect of temperature on acidophilus amylase production in SmF by *Aspergillus niger* RBP7.

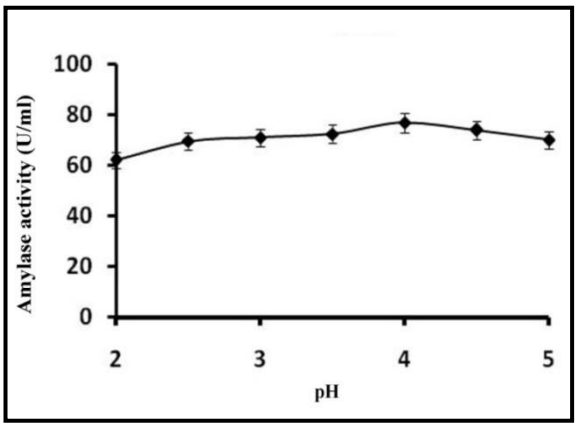

Fig 2.15. Effect of pH on acidophilus amylase production in SmF by *Aspergillus niger* RBP7.

2.3.5.2. Statistical optimization of submerged fermentation

Biodegradation of potato peel by *Aspergillus niger* RBP7 along with production of acidophilic amylase under submerged fermentation was carried out using RSM.

A total of 27 run with different combination of the four variables (in three equidistance level) were performed. ANOVA of the quadratic regression models suggested that the models were significant with a computed F value of 23.77 for amylase and P value (Prob>F) lower than 0.0004. The R^2 values were found to be 0.98 amylase production indicating that 97.83 % of the variability in the response could be explained by the model (Table 2.5).

Four important parameters were examined by 27 experimental run (Table 2.4). After analyzing data by Design-Expert, it was found that the effects of four factors, namely, potato peel 4.28 g (%, w/v), inoculums concentration 1.24 ml (%, v/v), pH 4.31, temperature 34.03 ^{o}C have highest impact on acidophilic amylase (131.96 U/ml) production ($P < 0.05$) by *Aspergillus niger* RBP7 (Fig. 2.16). Optimum levels of the four selected factors were again evaluated using Box-Behnken design (BBD) of RSM.

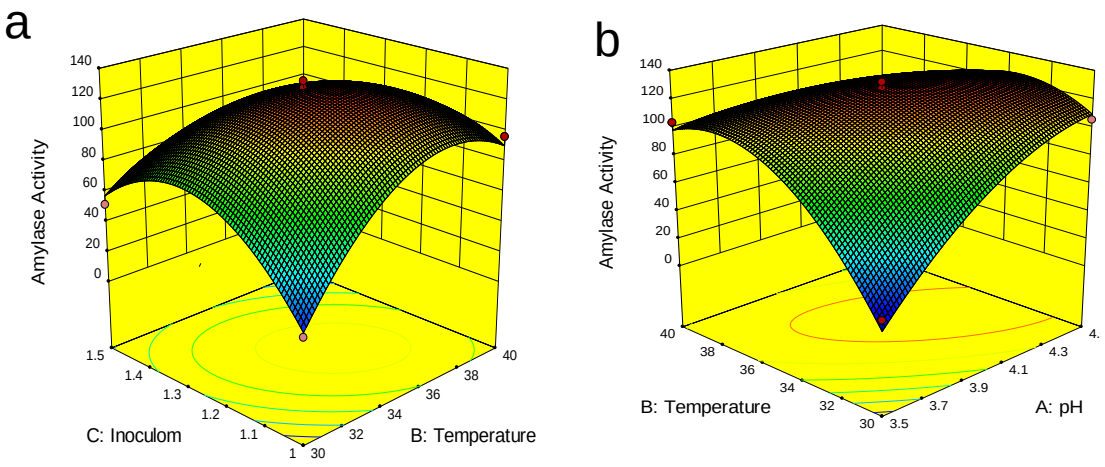

Fig.2.16. Response surface and three-dimensional contour plot of the combined effects of a) inoculums concentration (conidia/ml) and medium temperature (°C), b) temperature (°C) and medium pH on α-amylase production by *Aspergillus niger*RBP7 in SmF

Analysis of the quadratic effect indicated that all variables (potato peel concentration, inoculums concentration, pH and temperature) significantly contributed to these responses and the model also showed statistically significant. In OVAT maximum amylase production (115.8 U/ml) was observed at potato peel 4g/100ml, inoculums concentration 1.25 ml (%V/V), pH 4.0, temperature 35 ^{o}C, which was in good assessment with the predicted results (131.96 U/ml) of RSM. Therefore, the enzyme production was increased in RSM than OVAT due to the effect of interactions between four different variables.

Pathania et al. (2017) showed that speculate value (16.07 U/ml) is similar with actual value (16.07 U/ml) of amylase produced by *Bacillus amyloliquifaciens* where pH, temperature, inoculums size, incubation days and substrate concentration these five parameters were used for RSM.

Table 2.4. Experimental design used in RSM studies by using four independent variables each at three levels showing observed predicted values of acidophilus α- amylase production by *Aspergillus niger*RBP7 in SmF

Run	**Factor 1 A:pH**	**Factor 2 B:Temparature**	**Factor 3 C:inoculum**	**Factor 4 D:Substrate**	**Response 1 Amylase activity U/gds**	**Predicted value**
1	3.5	30	1.25	4	18.64	11.29
2	4	35	1.25	4	131.96	127.52
3	4	30	1.5	4	51.88	57.72
4	4	40	1.25	3	45.96	51.82
5	4	30	1.25	3	23.72	22.99
6	3.5	35	1	4	72.64	71.25
7	4	40	1.25	5	60.78	62.77
8	3.5	35	1.25	5	43.62	45.45
9	4.5	40	1.25	4	51.22	57.18
10	4	40	1	4	96.38	90.65
11	4	30	1	4	20.74	23.64
12	4	35	1.25	4	127.52	127.52
13	3.5	35	1.5	4	32.62	35.63
14	4	35	1.5	5	31.12	32.37
15	4.5	30	1.25	4	105.82	109.74
16	3.5	35	1.25	3	50.82	60.00
17	4.5	35	1.25	3	53.27	51.55
18	4	35	1.25	4	123.06	127.51
19	4	35	1.5	3	56.33	46.36
20	4.5	35	1.5	4	81.54	84.2
21	4.5	35	1.25	5	120.1	111.02
22	3.5	40	1.25	4	103.8	98.50
23	4	30	1.25	5	61.54	56.95
24	4	35	1	5	75.82	84.42
25	4.5	35	1	4	81.54	79.79
26	4	40	1.5	4	28.16	25.36
27	4	35	1	3	28.16	25.53

Table 2.5. Analysis of variance (ANOVA) for for acidophilic α- amylase production by *Aspergillus niger* RBP7 in SmF

Source	**Sum of Squares**	**Df**	**Mean Square**	**F- Value**	**p-value Prob> F**
Model	31313.97	14	2236.71	38.81	8.41E-08
A-pH	2446.735	1	2446.74	42.45	2.87E-05
B-Temperature	900.640	1	900.64	15.63	0.002
C-Inoculom	730.548	1	730.54	12.67	0.005
D-Subtrate	1512.457	1	1512.45	26.24	0.0003
AB	4883.214	1	4883.21	84.72	8.7E-07
AC	400.400	1	400.40	6.95	0.022
AD	1370.11	1	1370.11	23.77	0.0004
BC	2468.102	1	2468.10	42.81	2.75E-05
BD	132.25	1	132.25	2.29	0.16
CD	1327.509	1	1327.51	23.03	0.0004
A^2	2128.802	1	2128.80	36.93	5.52E-05
B^2	7844.876	1	7844.88	136.10	6.62E-08
C^2	8455.113	1	8455.11	146.69	4.36E-08
D^2	8759.884	1	8759.88	151.97	3.58E-08
Residual	691.674	12	57.63		
Lack of Fit	652.069	10	65.20	3.29	0.26
Pure Error	39.605	2	19.80		
Core Total	32005.65	26			

In SmF the result of optimized parameters are almost same with SSF except pH which is 3.0 in SSF and 4.0 in SmF. However temperature in SSF is 40 ^{o}C and 35 ^{o}C in SmF. It may

be due to the growth of microorganisms several metabolites are accumulated within the medium which change the initial pH of the medium. Normally in SSF condition moisture content as well as water activity is very low within the fermentation system. Thus, the chance of accumulation of metabolites is lower than the submerged fermentation as the mycelium penetrates the solid substrate. These effects have no major role to change the initial pH of the medium during fermentation. However, the accumulation of metabolites and change of pH during fermentation is very common in submerged state. These findings also reported by Samanta et. al. (2014) in bacterial system.

In case of temperature, for enzyme production, optimum temperature is 5 ^{o}C higher in SSF than SmF. This is due to higher metabolic rate in SSF. SSF occurs when fungal mycelium starts the breakdown of solid substrates by their hydrolytic enzymes. This higher metabolic rate is influenced by slight increase of temperature which increases the kinetic property of hydrolytic enzymes. As a result slightly higher temperature in SSF promotes the higher metabolic activity as well as enzyme secretion.

The rate of enzyme production in SmF through OVAT technique was 89.02 % lower as compared with SSF. Similar findings also observed in RSM study and production rate of α- amylase is 88.13 % lower in SmF comparison to SSF. Both these results indicated that the rate of amylase production was far higher in SSF, as the fungi are more potent for enzyme production in SSF. *Aspergillus niger* is a filamentous fungi; fungal mycelium are able to penetrate solid substances for nutrition uptake by secreting different types of hydrolytic enzymes including α- amylase. In case of submerged condition the hyphal propagation and enzyme secretion might be low.

SSF used in large scale production where as SmF mainly used in laboratory production (Vidyalaksmi et al., 2009). Holker et al. (2004) stated that SSF is more preferable than SmF in large scale production because of its lower catabolic repression, higher fermentation productivity, higher product stability etc.

2.3.6. Comparison between the enzyme production from *Aspergillus niger* RBP7 and *Aspergillus niger* MTCC 281

The standard strain *Aspergillus niger* MTCC 281 can produce 110 U/ ml amylase which was studied by Upadhyay and Pandey, (2009). The rate of production of amylase by *Aspergillus niger* RBP7 and *Aspergillusniger* MTCC 281were 131.96 U/ ml and 60.7 U/ml respectively. So, the newly isolated produces 2.2 fold more α- amylase than the reference strain. Comparison between amylase production by different MTCC strain and *Aspergillus niger* RBP7 was given in Table 2.6. In all cases the fungi produced amylase at acidic pH which is almost similar with the pH of RBP7.

Table 2.6. Comparison between amylase productions by different fungal strain

Organism	Enzyme production	Optimum pH	Optimum Temperature (°C)	Substrate	SmF/SSF	References
Aspergillus niger MTCC 281	110 U/ml		30	Wheat bran	SSF	Upadhyaya and Pandey (2009)
Aspergillus niger MTCC 104	1248 U/ml	4.2	70	Rice bran	SSF	Kumar and Duhan (2011)
*Aspergillus awamori*MTCC 9997	31.64U/gds	6	40	Cassava peel	SSF	Kalairasi and Parvatham (2015)
*Aspergillus niger*RBP7	131.96 U/ml	4.0	35	Potato peel	SmF	

2.4. Conclusion

Both SSF and SmF by RBP7 were carried out by following OVAT and RSM methods sequentially. Overall 1105.6 U/gds and 131.96 U/ml of α- amylase were produced by

RBP7 through RSM optimization in SSF and SmF respectively. The α- amylase production ability was compared with the reference strain *Aspergillus niger* MTCC 281. The newly isolated strain was better to produce α- amylase than reference strain and can be employed for its industrial exploitation.