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**Research Paper**

# Production of High Activity Amylase from *Trichoderma Reesei Ncim 1052* In Solid State Fermentation

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***ABSTRACT:-*** The fungus, *Trichoderma reesei* NCIM 1052 was used for the production of extra cellular amylase on agricultural residues viz. Soy cake, Wheat bran, Rice bran and Ragi (*Eleusine corocana* Gaertn.) in solid state fermentation (SSF). Maximum enzyme activity (324.45 IU g-1) was obtained in a medium having Soy cake as the substrate, moisture level 1:1 (distilled water) and incubation for 72 hours with Tween-80 (0.05%) as the extractant. During the fermentation, starch was hydrolyzed completely with a corresponding increase in the biomass and amylase activity within 3 days. The optimum pH and Temperature of the crude enzyme was 5.0 and 50 $^0$C respectively.

***Keywords:-*** *Amylase, T. reesei, SSF, soy cake.*

## I. INTRODUCTION

Microorganisms produce enzymes which are involved primarily in the degradation of macromolecules to micro units capable of being taken into the living cell. Out of 2000 various enzymes that have been described, only a few like amylolytic, cellulolytic, lipolytic and proteolytic enzymes, have wide industrial and biotechnological applications **(Pandey.A *et al.*, 1999)**. α-Amylase (E.C.3.2.1.1) is a hydrolase enzyme that catalyses the hydrolysis of internal α-1, 4-glycosidic linkages in starch to yield products like glucose and maltose. It is a calcium metalloenzyme i.e. it depends on the presence of a metal co factor for its activity **(Ajita Sundarram .T *et al.*,2014).**

Exoamylases attack the α-1→4 linkages only from the non reducing outer polysaccharide chain ends. Glucoamylases (γ-amylases) are enzymes breaking glucosidic bond to produce solely α-glucose and β- amylases are enzymes breaking every alternate bond to produce maltose (α-1,4-glucan). **(Susan Budavari *et al.*, 1996).**

α-Amylase is produced by several bacteria, fungi and genetically modified species of microbes. The most widely used source among the bacterial species is the *Bacillus spp*. *B. amyloliquefaciens B. licheniformis, B.subtilis, B.cereus ,B.megaterium,B.coagulans, B.polymyxa, Bacillus stearothermophilus*, are widely used for commercial production of the enzyme. α-Amylases produced from *Bacillus licheniformis, Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* show promising potential in a number of industrial applications in processes such as food, fermentation, textiles and paper industries **(Ajita Sundarram .T *et al.*, 2014).** Alpha amylase produced after solid state fermentation from *Bacillus subtilis* (ATCC 6633) appears to have potential in industries due to its thermal, pH and detergent stability **(Malty S *et al.*, 2015)**.

Fungal sources of α-Amylase are confined to terrestrial isolates, mostly to *Aspergillus* species and to only few species of *Penicillium, P. brunneum* being one of them *Penicillium fellutanum* has been used in the recent past to produce α-Amylase by submerged fermentation (**Erdal Serkan *et al.*, 2010**) *Penicillium expansum* MT-1 *Penicillium chrysogenum, Aspergillus niger, Aspergillus oryzae and Aspergillus awamori* most commonly used for commercial production of α-Amylase by Solid state fermentation using various substrates such as, Loquat *(Eriobotrya japonica* Lindley) kernels, Wheat bran, Wheat straw, Corncob leaf, Rye straw**.(Balkan.B *et al.*, 2007).**

Amylases are of interest for biotechnological applications and in Starch processing, Sugar industry, Paper industry, Textile industry, Alcohol industry, Detergent industry, Glucose industry, Bread and Chapatti industry, Aquatic and Terrestrial Animal Feed industry and sewage treatment **(Gupta.,R *et al.*, 2003).**

There are mainly two methods which are used for production of α-Amylase on a commercial scale. These are Submerged fermentation and Solid State fermentation. Submerged Fermentation, This is a traditional

method used for growing the microbes in a liquid medium in fermenters with all parameters well controlled. Advantages: Better availability of Nutrients including Oxygen. Better control of parameters like Temperature, pH Limitations and high energetic expenditures. Solid State Fermentation, is a process where microorganisms are grown on solid support substrate. These supports can be either natural like lingo cellulosic wastes or other inert supports like plastic foams. Advantages: More Economical and cost effective. Higher product yields Less waste water generation ,Reduced Contaminations and Easier recovery of the yield **(S. Bhargav *et al*., 2008 Couto, S. R. *et al*., 2006 and Guerra *et al*., 2003).**

The production of amylases in SSF is affected by a variety of physicochemical factors, including the type of nutrient composition of the substrate, incubation temperature, pH, aeration, concentration and the type of carbon, phosphate and nitrogen sources, concentration and age of the inoculum, particle size and moisture level of the substrate (**Balkan and Ertan, 2007; Rodriguez *et al*., 2001; Ramesh.M.V *et al*., 1990 and Sanroman, 2006).** Therefore, after selecting a culture medium for amylase production, the fermentation conditions must be optimized to improve enzyme production at a low production cost **(Balkan and Ertan, 2007; Spier *et al*., 2006)**.

The purpose of this work was to study the production of the enzyme, amylase by *Trichoderma reesei* in solid state fermentation and optimized the cultural conditions such like appropriate substrate, pH, Temperature and Moisture level.

## II. MATERIALS AND METHODS

**Cultivation of *T. reesei*: Inoculum development**

*T. reesei* NCIM – 1052 used in the present study was obtained from the National Chemical Laboratory (NCL), Pune. The culture was routinely maintained on a Potato dextrose agar medium at $4^0$C.

**Cultivation conditions**

*T. reesei* NCIM – 1052 was cultivated using a Potato dextrose broth at $28^0$C under constant agitation at 200 RPM on an orbital shaker.

**Development of seed inoculum**

A loopful of conidia of *T. reesei* NCIM – 1052 grown on Potato dextrose agar was inoculated into a 100 ml of Potato dextrose broth in a 500 ml Erlenmeyer flask and incubated for 5-7 days at 28-$30^0$C **(J.M.Khire *et.al*., 1991).**

**Solid state fermentation**

The initial enzyme production was checked individually by using various agro residues like Wheat bran, Soy cake, Rice bran and Ragi procured from the local market. Further optimization of the process parameters was studied using Soy cake as a substrate for SSF.

**Fermentation conditions**

All experiments were conducted in 500 ml Erlenmeyer flasks containing 10 g of agro residues (Wheat bran, Soy cake, Rice bran and Ragi) moistened with 10 ml distilled water were autoclaved at $121^0$ C for 40 minutes. Flasks were then inoculated with 1ml spore suspension containing $10^{-7}$ spores $ml^{-1}$ from a 5-7 days old culture grown on a Potato Dextrose broth. After inoculation, flasks were incubated at $28^0$C for uniform distribution of fungal spores in the medium. After every 24 hrs. a flask was harvested and its contents were extracted for amylase activity assay (**Pandey,A *et al*., 1999).**

**Enzyme Extraction:**

After solid state fermentation, 50 ml of 100 mM Acetate buffer (pH: 5.0), Tween- 80 (0.05%), Triton-100, distilled water, tap water and Nacl 0.85% (Saline) were added each to all the three flasks, each containing 10g fermented Soy cake. Flasks were kept on a rotary shaker operated at 200 rpm for 2 hrs. at room temperature for the extraction of enzyme from fermented Koji. At the end of extraction, the suspension was squeezed through double layer of muslin cloth and centrifuged at 5000 x g for 20 minutes at room temperature. The clear supernatant was used for the enzyme assay and enzyme preparation (**J.M.Khire and A.Pant *et al*., 1996.)**

**Amylase Assay:**

Amylase measurement was carried out at $50^0$ C. The reaction mixture consisted of 1% w/v Soluble Starch solution with 100 mM Acetate buffer (pH: 5.0). Enzymatic reactions were started by the addition of 50 µl of enzyme solution. After 30 minutes, freshly prepared solution of 3`5` Dinitrosalysilic acid (DNSA) was added

to terminate the reaction. Absorbance was measured at 540 nm to measure the amount of enzyme which secrets 1mM of reducing sugar (glucose) under standard enzyme assay condition **(Miller, G.L *et al.*, 1959)**. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments with 3-5% variability.

**Optimization of Important Parameters:**

**Amylase Production Using Different Agrowastes as Soild Substrate by *T. reesei* NCIM – 1052**

*T. reesei* NCIM – 1052 was cultivated on different substrates such as Wheat bran, Soy cake, Rice bran and Ragi. Four replicates, all growth conditions mentioned above were followed and only agro residues (10 g in each case) varied in all sets.

**Effect of Incubation Time:**

Effect of Incubation period on the amylase production was determined by the enzyme assay after $24^{th}$, $48^{th}$, $72^{th}$, $96^{th}$ and 120 h at $28^{0}$C.

**Effect of Moisture level:**

The effect of moisture level on enzyme production was evaluated by varying the ratio of soy cake to water at 1:1, 1:1.2, 1:1.5).

**Effect of surfactants:**

The effect of surfactants on enzyme extraction was evaluated using surfactants viz., Tap water, Tween- 80, 100 mM Acetate buffer (pH: 5.0), 0.85% Saline water, and Distilled water individually.

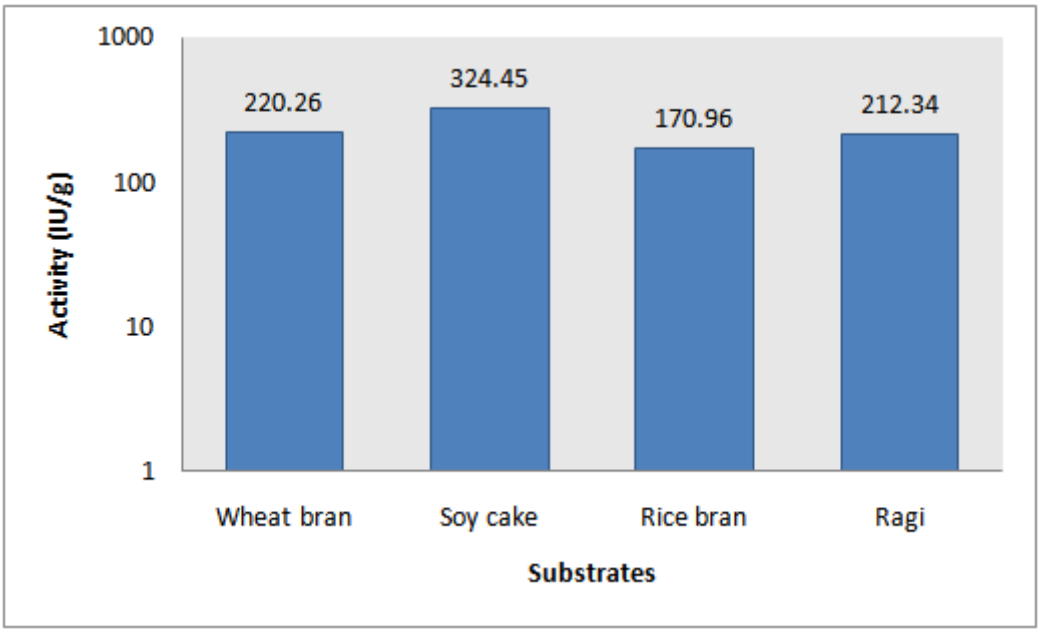
**Effect of Inoculum levels**

Effect of inoculum levels on the substrate was evaluated at varying concentrations including 1, 3, 5,10 and 15% (v/w).

## III. RESULTS AND DISCUSSIONS

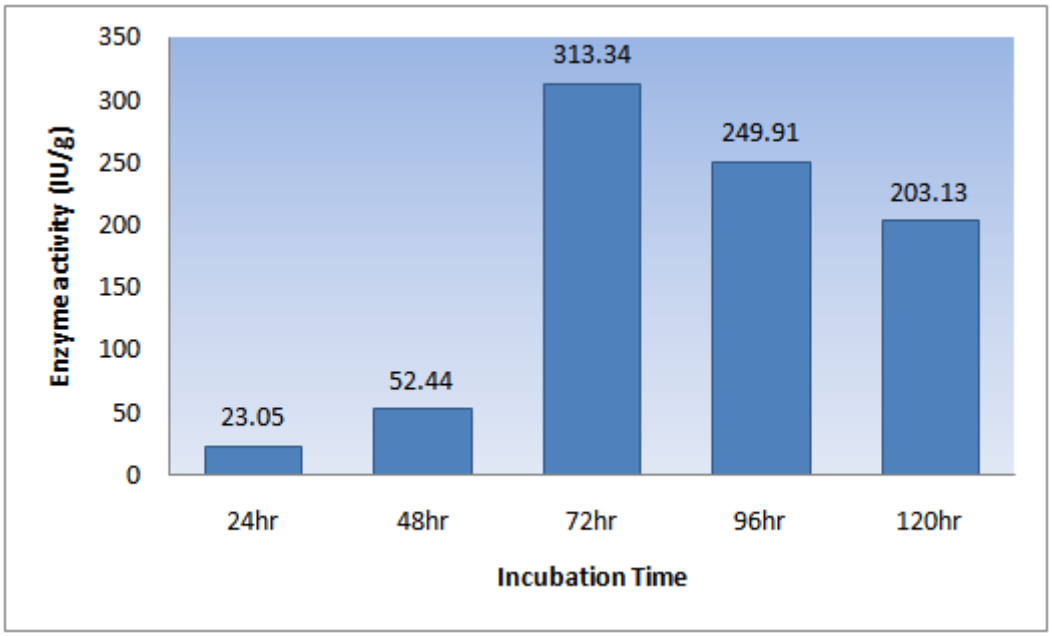
Amylase from fungi, Bacteria, Yeasts and agricultural residue sources has been produced by solid state fermentation as well as submerged fermentation **(Pandey *et al.*, 1999)**. To check the influence of various substrates on Amylase production during solid state fermentation, Soy cake medium was compared with other substrates as Soy cake is inexpensive and easily available than others (Graph 01) and incubated at room temperature **(Ramesh, M.V *et al.*, 1990)**. Our results shown that Soy cake is superior to other substrates for the production of Amylase enzyme.

**Graph 01: Role of solid substrates for the production of Amylase by *T. reesei* NCIM – 1052 in SSF**



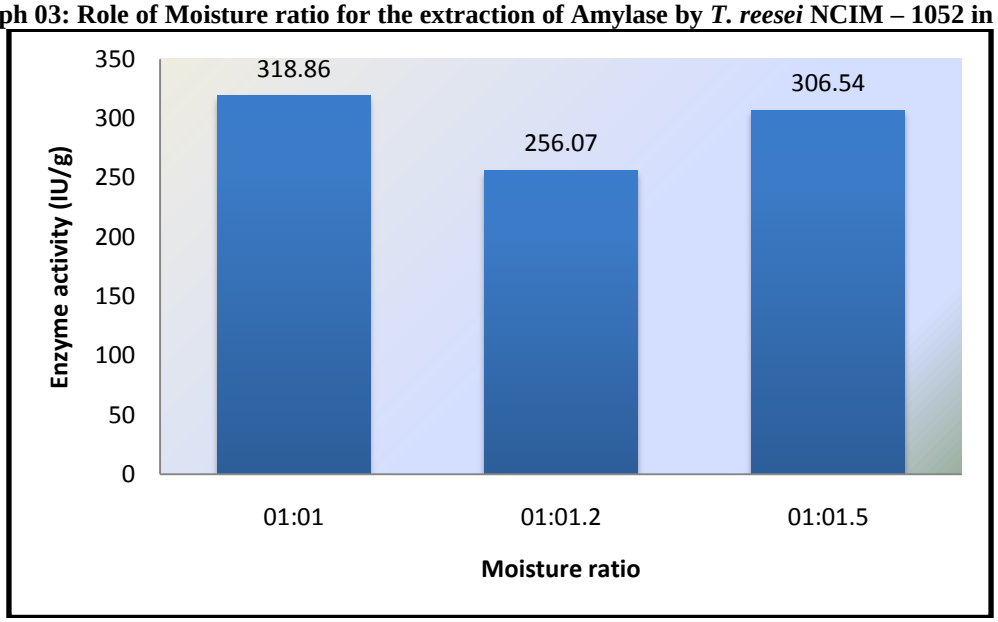
Low level of Amylase appeared in the early stages of incubation and enzyme levels reached a maximum by 3 days of solid state fermentation. A prolonged incubation time beyond this period did not help to further increase the yield (Graph.02). The duration of incubation depend on the growth rate and enzyme production pattern of the strain. The starch source of soy cake substrate was completely hydrolyzed after 3 days of incubation. The culture grew well and produced Amylase at 28 $^{0}$C.

**Graph 02: Role of Incubation Time for the production of Amylase by *T. reesei* NCIM – 1052 in SSF**




The moisture content in a solid state fermentation is a crucial factor that determines the success of the process. The importance of moisture level in solid state fermentation media and its influence on microbial growth and product biosynthesis may be attributed to the impact of moisture on the physical properties of the solid substrate fermentation.1:1 level of moisture ratio yielded highest enzyme activity of 318.86 IU/g (Graph.03).

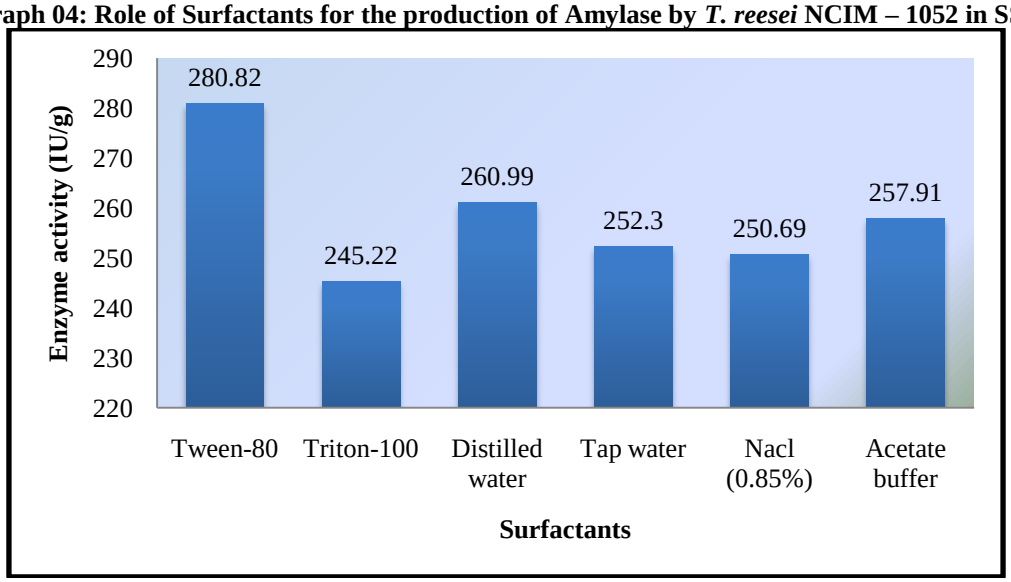
**Graph 03: Role of Moisture ratio for the extraction of Amylase by *T. reesei* NCIM – 1052 in SSF**




There have been reports on the use of 2% 100mM Acetate buffer (pH: 5.0), Distilled water, Tap water, Tween -80, as the extractants of Amylase from solid substrate used for the fermentation. But, in our studies it was observed that various salt solutions like 0.85% Saline water, Tap water, Distilled water at 2% can also be used to extract Amylase with a recovery of Amylase having 250.69 IU/g activity, 252.30 IU/g and 260.99 IU/g

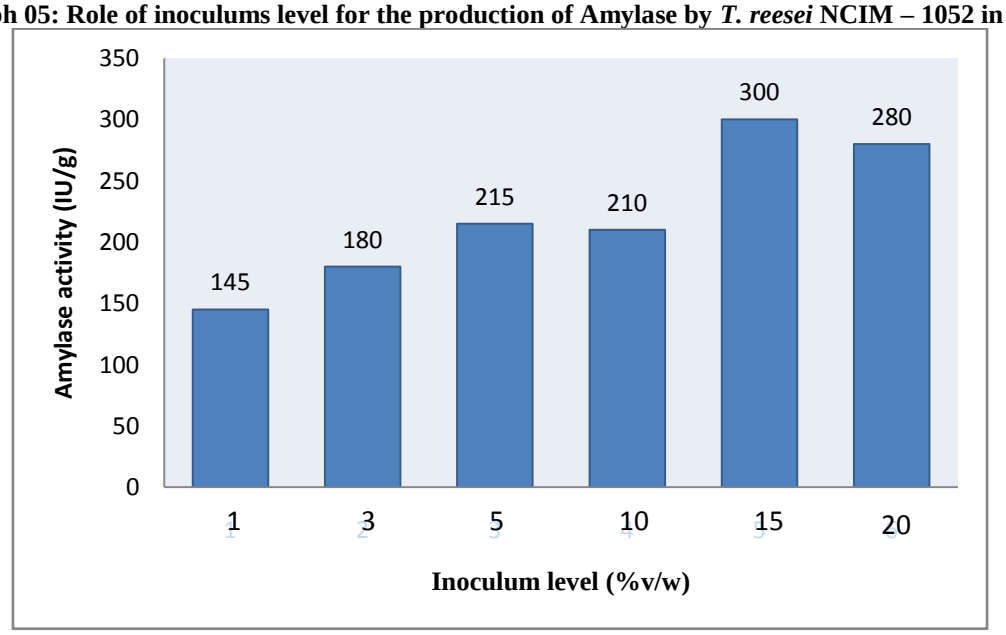
activity respectively. (Graph.04) However, 0.05% Tween- 80 was proved to be the best buffer solution amongst the all tested.

**Graph 04: Role of Surfactants for the production of Amylase by *T. reesei* NCIM – 1052 in SSF**




Inoculum level is an important factor for the production of enzymes. High inoculum concentration increases the moisture content to a significant extent. The free excess liquid present in an unabsorbed form creates an additional diffusion barrier together with that imposed by the solid nature of the substrate and lead to a decrease in growth and enzyme production **(Balkan *et al.*, 2011**). On the other hand, low inoculum level introduces a lower number of cells in the production medium. This requires a longer time to grow to an optimum number to utilize the substrate and form the desired product **(Kashyap *et al.*, 2002**).

**Graph 05: Role of inoculums level for the production of Amylase by *T. reesei* NCIM – 1052 in SSF**




Various inoculum levels (1, 3, 5, 10, 15 and 20 % (v/w)) were tried to study their effect on amylase production. The highest enzyme production (300IU/g) was obtained at 10 % (v/w) inoculum level (Graph.05). Similarly, Anto *et al.*, (2006) reported that varying inoculum size of bacterial cells during the fermentation indicated 10 % (volume per mass) inoculum as optimum for the enzyme production. Further increase in inoculum size was found to adversely affect the enzyme production.

## IV. CONCLUSION

Amylase from fungi, Bacteria, Yeasts and agricultural residue sources has been produced by solid state fermentation as well as submerged fermentation. In the present study, *T.reesei* NCIM 1052 has been used to produce Amylase in solid state fermentation, using different substrates like Soy cake, Wheat bran, Rice bran and Ragi etc. Different moisture levels, several incubation times and various inoculums levels were studied. Several buffers were used to extract Amylase. After studying the effects of substrate, moisture level, surfactant, incubation time and extractants; it was found that Soya cake, 1:1, Tween -80, 72 hours and acetate buffer pH: 5.0 were the optimum parameters in the case of *T.reesei* for the maximum Amylase production.

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