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Screening of agro-residues & factors influencing for the production of β-fructofuranosidase from *Aspergillus niger*

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Abstract
In the present research experiment we have used local & chiefly available four different waste-going agro-residues such as Rice bran, Wheat Bran, Bagasse, & Orange peel were collected from agriculture fields and powdered to obtain a particle size of 1.0 to 2.0 mm. SSF was performed with all the four substrates by a fungus, *Aspergillus niger* to observe potenial production of an extracellular enzyme, Beta –fructofuranosidase under the influence of various factors. Among all selected agro-residues, the rice bran with tap water was found most suitable substrate for higher production of enzymes under the fixed optimized conditions of 120 hours of incubation, 1000 µl of moisture level, 5th day culture of inoculum age, and at 35 ^{0}C of temperature.

Keywords: SSF, *Aspergillus niger*, agro-residues, β-fructofuranosidase, enzyme

1. Introduction
Enzyme is either protein or RNA, that catalyzes a specific chemical reaction. It does not affect the equilibrium of the catalyzed reaction, remains unaltered in the process. Enzymes are mainly classified into six major classes, such as i) Oxidoreductases, ii) Transferases, iii) Hydrolases, iv) Lyases, v) Isomerases & vi) Ligase. Some of the biological roles are wine manufacturing, cheese making, candy making, bread whitening, dissolving blood clot, wound healing, correction digestion, etc.

1.1 Beta –fructofuranosidase (Invertase)
B-fructofuranosidase is also called as Sucrase, Saccharase or 6-D-fructosidase. This enzyme has been found to occur in green leaves, fruits, grains, stems, potato tubers, some roots, pollen and lower plants as fungi and bacteria. Maximum activity is obtained with low concentration of sucrose (5-10%). Sucrase also hydrolyses the sugar genfianose, raffinose, stachyose and to some extent insulin. Invertase is yeast - derived enzyme, splits sucrose into glucose and fructose. The optimum temperature for Invertase is 140 ^{0}F (60 ^{0}C). The enzyme has excellent activity at lower temperatures, but reaction times are extended. Hence, it can be used at temperatures up to 140 ^{0}F without loss of activity. Above 140 ^{0}F activity begins to decrease. Invertase is used to prevent sugar crystallization in confections by hydrolysis of sucrose into glucose & fructose in fondants or chocolate coated candies with soft centers. In addition to its main confectionary application, it can be used to produce melibiose from raffinose or D- Fructose from inulin, since it contains b-fructosidase activity. Invertase can also be used in some specialty fruit juice products to decrease sucrose levels. It is used for the production of invert syrups, preventing crystallizing. Increasing sweetness and improving flavor and color characteristics of the invert sugar. However, the SSF process development was still in its infancy and was considered not suitable for many of the potential large-scale applications (Sudheerkumar, 2008) [2].

1.2 Agro residues
Over decades of years, it has become clear that research on the degradation of lignocellulose by fungi may lead to other industrial applications (Crawford and Crawford, 1980) [11].

Using of agroindustrial residues as substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or not-utilized residues (Dayanne *et al*., 2012) [12]. Agro-residue is the most potential fiber resource, which is helpful to sustainable development of composite industries. It is used as substrate for the production of various enzymes. These residues not only have cheap source of nutrients for fermentation to produce enymes but use of their waste-going nutrients can overcome the use of a synthetic medium which are very expensive and uneconomical. So they need to be replaced with more economically available agricultural and industrial by-products, as they are considered to be good substrates for SSF to produce enzymes (Rajshree & Rajni, 2016).

1.3 Solid-state fermentation (SSF)

Solid-state fermentation (SSF) is defined as the growth of microbes without free flowing aqueous phase (Bhargav e. al., 2008) [9]. Recent years solid-state fermentation (SSF) technique has been developed and used more extensively. It has advantages over other fermenation processes like simple technique, low capital investment, cheaper production of enzyme having better physiochemical properties, lower levels of catabolite repression and better product recovery (Baysal, *et al*., 2003) [5]. The term solid-state fermentation (SSF) denotes cultivation of microorganism on solid, moist substrates in the absence of an aqueous phase, that is, at average water activity (Dayanne *et al*., 2012) [12]. SSF is a process during which microorganisms are cultivated in the presence of a liquid phase at maximal substrate concentrations or on inert carriers. SSF holds tremendous potential for the production of enzymes. lt can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source. A large number of microorganisms, including bacteria, yeast and fungi produce different groups of enzymes. However, selection of a particular strain, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved. Fungi and yeast were termed as suitable microorganisms for SSF, whereas bacteria have been considered unsuitable (Chinn *et al*., 2007) [13]. It has been reported that a strain of *Aspergillus niger* produce about 19 types of enzymes (Ashok *et al*. The selection of a suitable strain for the required purpose depends upon a number of factors, in particular upon the nature of the substrate and environmental conditions. Agro–industrial residues are generally considered as the best substrates for the SSF processes (Kunamneni, *et al*., 2005) [5] and use of SSF for the production of enzymes is no exception to that. A number of such substrates have been employed for the cultivation of microorganisms to produce enzymes. Some of the substrates such as sugar cane, bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk etc., can be used for SSF. However, the selection of a substrate for enzyme production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate, and thus may involve screening of several agro-industrial residues. In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it, but also serves as an anchorage for the cells. The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate. However, some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates in such cases, it would become necessary to supplement them externally with these. It has also been a practice to pre-treat (chemically or mechanically) some of the substrates before using in SSF processes (e.g. lingo–cellulose), thereby making them more easily accessible for microbial growth.

Among the several factors that are important for microbial growth and enzyme production using a particular substrate, particle size and moisture level/water activity are the most critical. Generally, smaller substrate particles provide larger surface area for microbial attack and, thus, are a desirable factor. However, too small a substrate particle may result in substrate agumulation, which may interfere with microbial respiration/ aeration, and therefore result in poor growth. In contrast, larger particles provide better respiration/ aeration efficiency (due to increased inter – particle space), but provide limited surface for microbial attack. This necessitates a compromised particle size for a particular process. Different types of fomenters (bioreactors) have been employed for various purposes in SSF systems. Laboratory studies are generally carried out in tray–drum or deep–rough type fomenters. The development of a simple and practical fomenter with automation is yet to be achieved for the SSF processes.

1.4 *Aspergillus niger*

Kingdom	:	Plantae
Division	:	Mycota
Sub- Division	:	Eumycotina
Class	:	Ascomycetes
Sub- Class	:	Euascomycetidae
Series	:	Plectomycetes
Order	:	Eurotiales
Family	:	Eurotiaceae
Genus	:	Aspergillus
Species	:	niger

Aspergillus niger is a fungus and one of the most common species of the genus Aspergillus. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil. Many useful enzymes are produced using industrial fermentation of *Aspergillus niger*. The processes provide extracellular fungal enzymes and have been the basis to initiate the microbial enzymes production by SSF in industrial environment (Sudhirkumar *et al*., 2008) [2] *Aspergillus niger* fermentation is "generally regarded as safe" by the United States Food and Drug Administration. In 2006 it was reported that a secreted RNase produced by *Aspergillus niger* called actibind has antiangiogenic and anticarciogenic characteristics. It is also cultured for the extraction of the enzymes glucose oxidase and Alpha–galactosidase. It is less likely to cause human disease than other Aspergillus species, but if large amounts of spores are inhaled, a serious lung disease, aspergillosis can occur. *Aspergillus niger* is a fungal strain propagated on potato dextrose agar medium (PDA) at 30 ^{0}C and maintained at 4 ^{0}C. Therefore, the main objectives of present research experiment were, screening of chiefly & cheaply available raw agro–residues as suitable substrate for maximum production & activities of enzyme, β–fructofuranosidase of *Aspergillus niger* & to minimize agro-organic wastes to prevent environmental pollution.

2. Materials & Methods

2.1 Substrate used in SSF

Since different agro-residues have varied quanto-quality of nutrients, they can be used as substrate for *A. niger* in the SSF to induce production of varied degree of enzymes. Hence, four different locally & chiefly available agro residues such as Rice bran, Wheat Bran, Bagasse, & Orange peel were collected from agriculture fields of Bidar and powdered to obtain a particle size of 1.0 to 2.0 mm. SSF was performed with all the four substrates and their enzyme production was checked by assay.

2.2 Spore collection: The spore form of *Apergillus niger* collected from Department of soil testing, Agriculure Research Centre, Janawada, District Bidar. They where sub-cultured on PDA medium.

2.3 Preparation of potato dextrose agar (PDA): 200gms of Potato & 20gms of dextrose were dissolved in distilled water and made to the final volume 1000 ml while adjusting the pH of 7.0. Then 20gm of agar was added, sterilized by autoclave to get clear solution.

Constituents	gms / ltr
Potato	200
Dextrose	20
Agar	20
Distilled water	1000 ml
pH	7

2.4 Sodium acetate buffer (0.05 m): 4.1gms of sodium acetate dissolved in 2.88ml of acetic acid, then final volume of 1000ml was made by distilled water to get 0.05M sodium acetate buffer and pH was adjusted to 5.

2.5 Slant preparation: 5ml of PDA medium was poured into the test tube and allowed it to solidify. Then, *Aspergillus niger was inoculated into it* and kept it 3 days for incubation at 37 ^{0}C.

2.5 Preparation of spore suspension: 5ml of sterilized normal saline (0.85% NaCl) added to the test tube slant containing cultures of *Aspergillus niger* to prepare spore suspension.

2.6 Preparation of solid-state fermentation (SSF) medium

Constituents	gms / Lt
$(NH_4)_2 SO_4$	45
$KH_2 PO_4$	23
Fe SO_{44}	7
Mg SO_4	0.1
Sucrose	50
Urea	11
Yeast extract	5
Distilled water	1000 ml
pH	5

All the above constituents were dissolved into distilled water & made the final volume to 1000ml by adjusting the pH at 5.

2.7 Preparation of DNS: 1gm of DNS, 30gms of sodium potassium tartarate & 1.6gm of NaOH dissolved in 100 ml of distilled water.

2.8 Preparation of standard sucrose solution & curve

100 mg of sucrose is dissolved in 100 ml of distilled water.

S. No	Std. sucrose (ml)	Distilled water (ml)	Incubate at 37 ^{0}C for 10 min	DNS (ml)	Keep in boiling water bath for 10 min	Distilled water (ml)	Optical Density at 545 (nm)
1	0	1		1		8	0.08
2	0.2	0.8		1		8	0.15
3	0.4	0.6		1		8	0.25
4	0.6	0.4		1		8	0.34
5	0.8	0.2		1		8	0.43
6	1.0	0		1		8	0.51

2.9 Method of solid-state fermentation: 5gm of substrate & 5gm of SSF medium were mixed well, Then, plugged with cotton and autoclaved at 121 ^{0}C for 20minues at 15lb pressure. 0.5 ml of spore suspension inoculated asepectically into the conical flask containing autoclaved medium. The conical flask was kept for 3 days incubation at 37 ^{0}C, and then 25 ml of sodium acetate was added as buffer. The conical flask was kept on shaking incubator for 1hour at 150 rpm for homogenous mixing. Then, the solution was filtered using masculine cloth. This filtrate (as crude enzyme) is used for enzyme assay.

2.10 enzyme assay by DNS method

Enzyme (ml)	Sucrose (ml)	Incubate at 37 ^{0}C for 10 min	DNS (ml)	Distilled water (ml)	Keep in boiling water bath for 10 min	Distilled water (ml)	O.D. (545nm)
0.1	0.2		1	0.7		8	0.65
0.1	0.2		1	0.7		8	0.68
0.1	0.4		1	0.5		8	0.66
0.1	0.4		1	0.5		8	0.7

0.1ml of enzyme extract was taken into each of 4 test tubes and 0.2 ml of sucrose to 1st & 2nd test tubes and 0.4ml in last 2 test tubes. They were ncubated at 37 ^{o}C for 10 minutes. 0.1ml of DNS added to each of 4 tubes & finally distilled water was to all tubes to make total volume of 2 ml. Then they were incubated in boiling water bath for 10minutes. Again 8 ml of distilled water added to each of all test tubes before measurement of optical density at 545nm.

The sucrose is hydrolysed into glucose and fructose by the enzyme present in the crude mixture. Sucrose is usually digested by the enzyme Beta–fructofuranosidase. It, when measured under spectrophotometer for its absorbance gives

readings, indicating that the crude mixture of enzymes contains the enzyme, Beta fructofuranosidase which is responsible for digestion of sucrose. Hence it is concluded based on action of Beta – fructofuranosidase present in the crude mixture of enzyme on sucrose substrate. The enzyme activity in fermented substrate was calculated using following method.

Activity = µgm of maltose liberated x 2 x dilution factor.
Mol. wt. of maltose x incubation time

3. Results & Discussions

Table 1: Various substrates used for fermentation by *Aspergillus niger* & its enzyme activity

S. No	Substrate	Conc. (µgm/lt)	Activity (µmol/ml/min)
1	Bagasse + Tap water	1410	0.824
2	Orange peel + Bagasse + Tap water	840	0.491
3	Bagasse + Orange peel + Medium	1120	0.654
4	Rice bran+ Medium	1340	0.783
5	Wheat bran + Tap water	1460	0.850
6	Rice bran + Tap water	1560	0.912

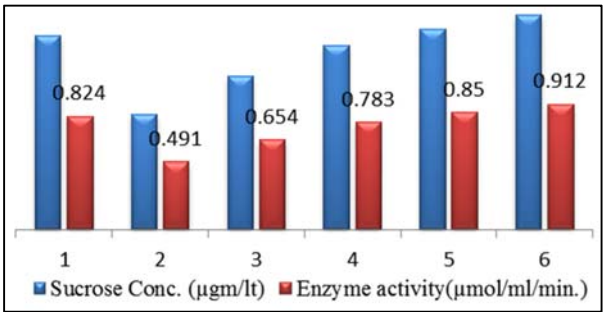

Fig 1: Agroresidues with sucrose concentration & enzyme activity

The production of the enzyme beta fructofuranosidase is achieved with all selected agro- residues as substrates for fermentation, however, its rate of production & activity varied with substrates (table 1 & fig. 1) as these were having different quanto-quality of nutrients. Type of medium also affects on the screening of agro – residues for fermentation (Rajshree & Rajni, 2011) [3]. It was observed that the production of enzyme was higher in tap water that in SSF, when used as medium along with agro-residues. Fermentations done with substrates in different combinations (wheat bran, rice bran, bagasse and orange peel were used either alone or in combinations) shown different activities. Among all selected agro-residues, Rice bran was found to be a good substrate for the production of Beta- fructofuranosidase. Further, the production of the enzyme was found to be higher in Rice bran with tap water than with SSF when used as medium. Beta – fructofuranosidase production is best achieved at 35 ^{0}C with moisture level of 1000 µlt and inoculum age of 5th day and incubation period of 120 hours.

3.1 Factors affecting on screening of agro-residues for the activity of fungus & production of enzymes

There are different factors such as temperature, dilution, type of medium, time of incubation, substrate, age of inoculum etc., affect on screening of agro-residues for the activity of *A. niger* & its enzyme production.

3.1.1 Temperature

Temperature and pH are known to be important parameters in the production of enzymes hence, the thermal stability of the enzyme, which is a function of the exposure time, must also be taken into account (Rajshree & Rajni, 2011) [3]. Therefore, temperature is one of the mojor factors affected on screening of agro–residues. It has played crucial role on the activity of enzyme. Usually the fermentation carried out at optimum temperature of microorganism favors greater metabolic activity and *Aspergillus niger* has shown its maximum activity at temperature 35 ^{o}C (table 2 & fig.2).

Table 2: Affect of temperature on the activity of enzyme

Temperature (^{0}C)	Activity (µM/min/ml)
30	0.41
35	0.82
40	0.63
45	0.43
50	0.38
55	0.35

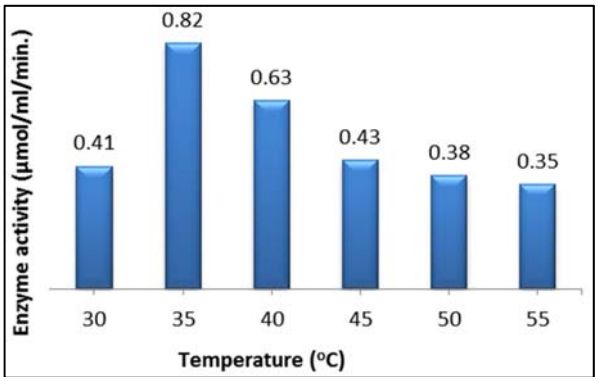

Fig 2: Effect of temperature on the production of enzyme in Rice bran + Tap water.

3.1.2 Inoculum age

Fermentation carried out with different aged cultures has shown varied activity (table 3 & fig.3), when fermentation carried out on 1st day culture has shown least activity and considerably activity increased up to the culture of 5thday, and 5th day culture has shown maximum activity. Beyond 5th day age culture, the activity decreased with increase in age.

Table 3: Affect of age of inoculum on the activity of enzyme

Age of inoculum (Days)	Activity (µmol/ml/min.)
2	0.05
3	0.10
4	0.15
5	0.20
6	0.18
7	0.15

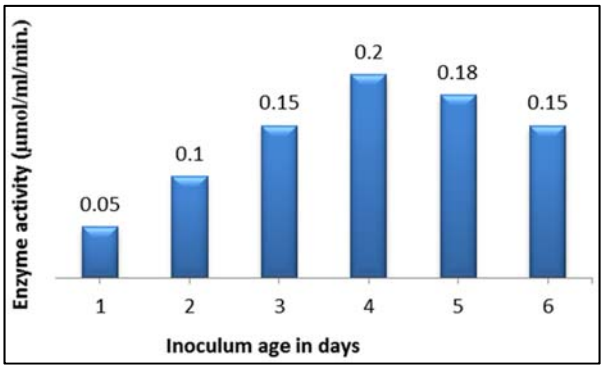

Fig 3: Affect of inoculum age on the production of enzyme in Rice bran + Tap water.

Lower inoculum age results in 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) [8]. Whereas, Anto *et al*. (2006) [1], have reported that increase in inoculum size adversely affected the enzyme production.

3.1.3 Moisture level

Fermentation carried out at different moisture level has shown significantly varied affects table 4 & fig.4). It was seen that, fermentation of substrate with moisture level of 1000µlt has maximum activity than other level of moisture. Further it was also observed that raise & less in moisture level of substrate has decreased the activity of fungi, it may be due to lower

Table 4: Affect of moisture level on the activity of enzyme

Moisture level (µlitre)	Activity (µmol/ml/min.)
500	0.05
1000	0.30
1500	0.20
2000	0.25
2500	0.15
3000	0.10

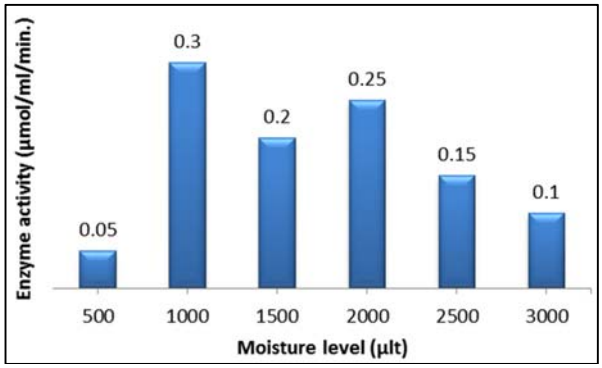

Fig 4: Affect of moisture level on the production of enzyme in Rice bran + Tap water

moisture content causes reduction in solubility of the nutrients of the substrate, low degree of swelling and high water tension as reported by Balkan & Ertan (2007) [6], and higher moisture levels can cause a reduction in enzyme yield due to steric hindrance of the growth of strain by reduction in porosity (interparticle spaces) of the solid substrate, thus interfering with oxygen transfer (Perez-Guarre, *et al*. 2003) [7].

3.1.4 Incubation period

It was observed that, fermentation carried out for different incubation period shown notably increased activity from 48–120 hours, beyond this range (i.e. from 120-168 hours) activity was reduced. Production of enzyme is however, maximum for 120 hours of fermentation (table 5 & fig.5). Similar results with *A. niger* observed by Dayanne *et al*., (2012) [12] that, the SSF with rice husks and wheat straw showed higher enzymatic activities over the time & all the trials showed the concentration of total enzymes activities were higher in later days than in earler days..

Table 5: Affect of Incubation period on the activity of enzyme

Incubation period (hours)	Activity (µmol/ml/min.)
48	0.24
72	0.45
96	0.65
120	0.85
144	0.74
168	0.68

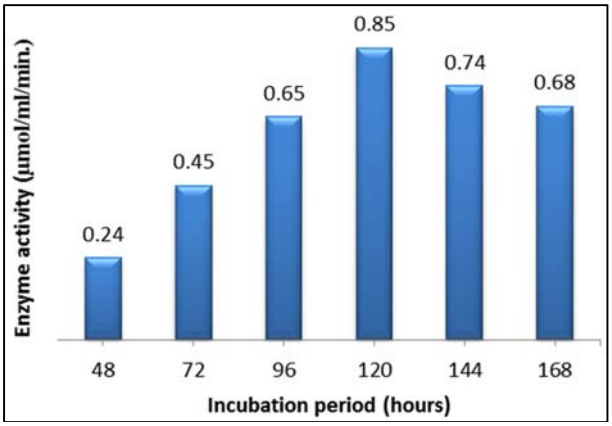

Fig 5: Affect of incubation period on the production of enzyme in Rice bran + Tap water.

3.2 Optimization of medium for *Aspergillus niger*

Optimization of the proper substrate and additives are an important part of the process. The optimized conditions fixed for the maximum production & activity of enzyme of *Aspergillus niger* were 120 hours of incubation, 1000µl of moisture level, 5th day culture of inoculum age, and 35 ^{0}C of temperature.

4. Conclusions

On one hand, by utilizing the low cost agricultural residues, SSF adds on to economic feasibility of the process (Robinson and Nigam, 2003) [14] and on other hand it solves the problem of its disposal which otherwise cause pollution (Singhania *et al*., 2009) [15]. Thus SSF is considered as most ecofriendly process because it uses agro-organic wastes as raw materials for the production of enzyme. Solid-state fermentation is better suited for the production of crude mixture of various enzymes by using fungi. It is simple & cheap process hence can be handled easily in the laboratory for enzyme production using *A. niger*.

Among all selected agro-residues, the rice bran with tap water was found most suitable substrate for higher production of enzymes under the fixed optimized conditions of 120 hours of incubation, 1000µl of moisture level, 5th day culture of inoculum age, and at 35 ^{0}C of temperature.

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