



Novel Fungal Co-Culture Technique for Enhanced Bioconversion of Agro-Industrial Waste to Amylase

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Abstract

Global strategies for management of solid waste occasionally lead to the environmental pollution. Now a days environmental friendly methods to valorize this waste are more demand to reduce the global warming. Solid-state fermentation (SSF) which is a potential waste recycling method to convert these solid wastes into value-added products by microbial population. In the present study, SSF was carried out using four substrates namely castor husk, rice husk, groundnut fodder, sugarcane bagasse and saw dust for the selection of renewable and chief substrate for the industrial production of amylase enzyme. We used two indigenous strains i.e., *Aspergillus protuberus* and *Aspergillus unguis*. Maximum production of α -amylase 1.614 U/g of substrate and 0.958 U/g of substrate on 2nd day of incubation in rice husk respectively. Groundnut waste (0.847 U/g of substrate) and castor husk (0.692 U/g of substrate) were also showed highest production of glucoamylase on 1st day and 2nd day of incubation in SSF. Further, prominent increase in the production of α -amylase (12 U/ml) and glucoamylase (3 U/ml) and extra cellular secretion of protein (20 mg/ml) was noticed in co-culture system on 2nd day of incubation in submerged fermentation (SmF).



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Introduction


Cultivation plays a vital role in India's economy and 54.6% of total workforce is connected in cultivation and similar fields¹ and accounts for 17.8% of global Gross Value Added (GVA) for year 2019-20. Maximum wastes that are generating from these industries

are inedible and produces by many activities such as crop harvesting, animal husbandry and others. According to global annual statistics estimated that the production of rice straw, wheat straw, corn straw, sugarcane bagasse and rice husk generation at 731, 354, 204 181 110 Mt respectively.² Though

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wood biomass squander was put at 4.6 Gt/Yr. waste from coffee and olive oil industries were assessed at 7.4 and 30 Mt/Yr. Almost 80% of the total wastes obtained from cereals and sugar cane in total biomass.^{3,4,5} The possible way for the disposal of these wastes are burning in fields or using for landfills in turn which creates many environmental problems. Now a days researchers are exploring these waste materials for production of important compounds in chief and cost effective manner.

The major biological activity of amylase enzyme is to catalyze starch into glucose, maltose and maltodextrines and classified under the family of GH13, with eight subfamilies.⁶ Apart from this it also engaged in other physiological phenomenon that include germination, defenses and development.⁷ It is a complex enzyme which includes three enzymes, i.e., α -, β and glucoamylases. Amongst these α -amylase has broad advantages in many industries viz., pharmaceutical, food, textile, paper, and detergent⁸ (Fig.1) due to its thermal and pH stability.⁹ Commercial production of microbial enzymes showed approximately 30% of the global market.¹⁰ Generally many microorganisms have the potential to secrete α -amylase enzyme, among them fungi with maximum potential and

appropriate candidate for industrial production. These fungi have the ability to utilize low-cost renewable substrates as a medium for growth, ease of enzyme recovery, pH stability, temperature, and less cofactor.¹¹ Because of low water, less energy utilization and high enzyme yield SSF is more preferable than the submerged fermentation for the commercial production of enzymes.¹²

In general the most important limiting factor in present usage of commercial enzymes manufacturing methods is their cost. Growth medium will determine the 30–40% cost of many industrial enzymes.¹³ Current years, the usage of lignocellulosic biomass in biorefineries as a renewable waste for the generation of bioenergy, chemicals, and enzymes is has gained strength.¹⁴ Effective utilization of this waste as cheaper source for the manufacturing of commercially industrial enzymes would not only be useful in decreasing the enzyme cost but also in increasing the value of underutilized substrates. Therefore, increasing the tribal economy with solving dual purpose in mutualistic method. Exploitation of various solid substrates for production of a enzymes with different fermentation methods is well studied.^{15,16} However, the hunt for novel substrates for cost-effective production of more robust enzymes is still going on.

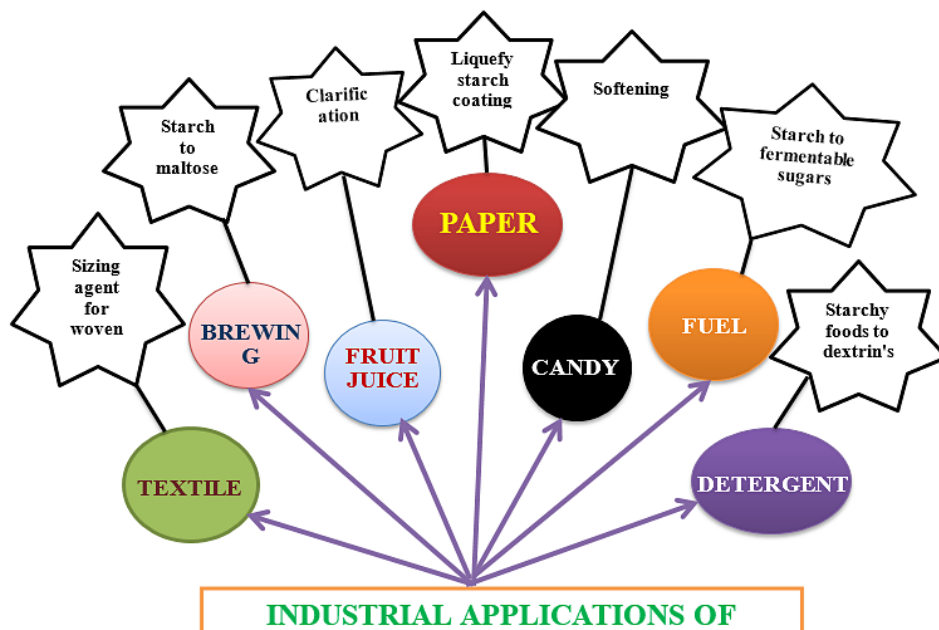


Fig.1: Amylase applications in different industries

Table 1: General agro-industrial wastes used as a renewable substrates used in SSF for valorization to value added products.

S. No	Renewable Substrate	Source Organisms			Product
		Bacterial Co-Culture	Fungal Co-Culture	Both Bacteria And Fungi Co-Culture	
1.	Apple pomace		<i>Aspergillus ornatus</i> , <i>Alternaria alternata</i>		Citric acid
2.	Sweet potato flour		<i>Trichoderma</i> sp and <i>Sacharomyces cerevisiae</i>		Bioethanol
4.	Wheat bran	<i>Bacillus thuringiensis</i> and <i>Bacillus cereus</i>			Eupenicillium
5.	Palm kernel cake			<i>Bacillus-Tricoderma</i>	cellulase
6.	Corn cob and Bermuda grass		<i>Tricoderma reesei</i> and <i>Aspergillus niger</i> GS1		Amylase, Fpase and Xylanase
7.	Saw dust and Wheat bran		<i>Cladosporium Sphaerospermum</i> , <i>Aspergillus flavus</i> and <i>Epicoccum purpurascens</i>		Xylanase
8.	Wheat bran		<i>Aspergillus penicillioides</i> and <i>Aspergillus flavus</i>	Xylanase, Fpase,	β -xylosidase CMCase.
9.	Wheat bran pulse husk and mustered peel		<i>Trametes hirsute</i> and <i>Phaenerochaete</i> sp.		Laccase, Pectinase
10.	Pineapple peel banana peel, and papaya peels		<i>Phaenerochaete chrysosporium</i> and <i>Scizophyllum commune</i>		α -Amylase and Cellulase

Apart from this the other approaches for the enhanced production of important enzymes is use of chemical inducers, the use genetically modified microorganisms, or the co-culture methods are important options. Use of chemical inducers and genetically modified bacteria having its own disadvantages. Co-culturing microbes were further evolved, and found better option for the production of many industrially important products such as pharmaceuticals, nutraceutical, nourishment, and drinks on a huge scale.^{17,18} It also plays outstanding role in the bio-remediation and bio-

energy divisions^{19,20} Co-culture system was its own advantages over the mono cultures i.e., flexibility, vigor, and ability to attempt modern errands and has immense potential for biotechnological applications.²¹ Moreover, artificial co-culture methods overcome obstacles of monocultures or consortia with additional focal points in investigating allelopathic relations²² in nourishment industries with fermentation²³ and medicate disclosure.²⁴

The substrates listed in table 1 are easily available and also a nutrients rich agro industrial waste

materials which can be used as substrates in SSF. The choice of considering pretreated substrates for enzyme production has an impact on the outcome. Many fungal strains have the ability to produce α -amylase. The glucoamylase was produced by different organisms like *Aspergillus awamori*, *A. saitoi*, *A. oryzae*, *Rhizopus* sp, *Mucor* sp, *Penicillium* sp., and *Yeast*.²⁵ Hence, the present study was intended to choose natural waste as cheaper source for the production of robust and low cost amylase enzyme suitable for industrial applications. Further, production capacity of amylase enzyme among the two indigenous *Aspergillus* strains individually and in co-culture methods using the SSF and SmF of natural agro-industrial waste as carbon source.

Materials and Methods

Microorganism

An indigenous two *Aspergillus* strains (*Aspergillus unguis* Accession number KX816008 and *Aspergillus protuberus* Accession number KX427028) were evaluated for their capability to utilize solid waste as a carbon source for its multiplication and production of amylase under SSF conditions. Czapek Dox agar medium was used to maintain pure cultures at $30 \pm 2^\circ\text{C}$ and maintain at 4°C .

Inoculum Preparation

10-15 ml of sterile distilled water with 0.8% tween-80 was added to the slant which was well speculated and shaken vigorously, then the spore count was adjusted to 1×10^7 spores/ml with fresh sterile distilled water and used to carry out solid state fermentation of different substrates.

Plate Screening Method

The selected fungal strains were spot inoculated on the medium which is supplemented with starch and incubated at $30 \pm 2^\circ\text{C}$. Dominant growth was observed for both the cultures at 48h of incubation and 1% iodine solution was poured on to the culture plate and incubated (Karri *et al.* 2014). Vanishing of blue color surrounding the colony indicated the production of extracellular amylase enzyme which break down the starch in to glucose and utilized for the physiological activities. In the culture medium where the starch is not degraded showed in the blue color only.

Lignocellulosic Substrates

Commonly available local agro-industrial waste substrates were collected, dried under controlled temperature and ground in to fine powder individually and sieved with a 2 mm screen to get even particle size. In the present study we included rice husk, groundnut fodder, saw dust, sugarcane bagasse and castor husk were chosen.

Solid State Fermentation

250 ml Erlenmeyer flasks was used to carry out Solid state fermentation (SSF) which contains 10 grams of different lignocellulosic substrates in each flask. Czapek Dox broth with the composition of two gm of NaNO_3 , one gm of K_2HPO_4 , 0.5 gm of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 gm of KCl, 0.01 gm of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 30 gm of sucrose and 5 gm of starch. 50% moisturization was maintained in each carbon source by adding different volumes of (10-15ml) sterile dH_2O and sterilized at 121°C for 15 min. Medium was inoculated with specified volume of fungal spores and incubated at ambient temperature and samples were collected at every 24 h for enzyme estimation by slightly modified Miller²⁸ method.

Co-Culture Method By Submerged Fermentation

In co-culture system SmF was done in 250 ml conical flasks. Ten grams of rice husk was used as a substrate and maintained 50% moisture level by adding 10 ml of distilled water and then sealed with cotton plug and autoclaved. 7-day-old mycelia were used for the preparation of the inoculum. Different spore ratios (1:1, 1:3, 3:1 and 0.5:0.5) were inoculated and incubated at ambient temperature ($30 \pm 2^\circ\text{C}$) 100 rpm/ min for 10 days. The samples were collected at every 24 hours for processing.

Enzyme Extraction

Amylase enzyme was recovered from the fermented substrate by adding different volume of sterile distilled water and shaking for 30 min in shaking incubator at a solid to substrate ratio of 1:10, then the extract was filtered with whatman no 1 filter paper and centrifuged at 5000 rpm for 20 min to obtain clear filtrate^{26,27} and used as a crude enzyme to measure its activity by spectrophotometric method.

Enzyme Assay

One International Unit is expressed as one μmol of glucose equivalents released per minute per ml. Appropriate dilutions were used in assessment of enzyme activity. Alpha and glucoamylase activity was estimated by the protocol of Miller.²⁸

alpha Amylase Activity

1% soluble starch was used as a substrate in 0.1 M citrate buffer pH 5.0. Freshly prepared culture filtrate was used as an enzyme source. The alpha amylase activity was measured by adding 1 ml of diluted enzyme solution and 1 ml of buffer solution along with the appropriate amount of substrate in a clean glass tube and incubated for 30 min at 45°C. After incubation period 3 ml of 3, 5- dinitrosalicylic acid (3,5-DNS) reagent was added to the tube to stop the reaction and tubes were boiled for 15 min in boiling water bath. Before cooling to room temperature, 1 ml of 40% Rochelle salt was added to each tube to preserve its color. Final volume in all tubes was made up to 7 ml with distilled water. Absorbance was measured at 575 nm with UV-Visible spectrophotometer considering 0.1 M citrate buffer as a reference blank and compared results with standard curve.

Glucoamylase Activity

1% maltose in 0.1 M citrate buffer (pH 5.0) was used as a substrate for the estimation of glucoamylase activity at 45°C for 30 min. The enzymatic reaction was carried out by pipetting 1 ml of diluted enzyme and one ml of citrate buffer in appropriate amount of

substrate and incubated at 45°C for 30 min. 3 ml of 3, 5-DNS reagent was added to the test tube to stop enzymatic reaction and the contents of the tube were heated for 15 min in boiling water bath. Activity was estimated as mentioned in alpha amylase activity.

Protein Determination

Lowry method²⁹ was employed for the estimation of ES proteins secreted by two strains into the culture medium after specific incubation periods.

Statistical Analysis

Triplicates were analyzed by using Descriptive Statistics in MS-EXCEL-2007 software.

Results and Discussion

A. protuberus and *A. unguis* were indigenous fungal cultures that were isolated from the Mahanandi forest soil sample and soil samples procured from the Kadapa cotton ginning mill respectively and both of these cultures were well known for hyper cellulase enzyme production. In this article, these cultures were compared for their ability to produce another industrially important enzyme, i.e., amylase, in a co-culture system and also screened the agro-industrial waste for the selection of suitable substrate for low cost production of the amylase enzyme. The results of screening of fungal cultures are shown as amylase production on plates. Maximum zone of starch hydrolysis was seen on the plates of *Aspergillus protuberus* and *Aspergillus unguis*, indicating that they are amylase producers (Fig. 2a, b).



a) *Aspergillus protuberus*



b) *Aspergillus unguis*

Fig. 2: Zone of inhibition shown by *Aspergillus protuberus* (a) and *Aspergillus unguis* (b).

Screening of Agro-Industrial Wastes

Globally (urban and rural locations), clearance of solid waste is a troublesome and persistent problem. The general way of disposal of this solid waste is incineration or landfill dumping. In recent years several scientists were studied on generation of important products from this waste in order to reduce the natural issues.³⁰ Several value-added products were generated from the glycerol waste which is produced from biodiesel industry, i.e., Organic acids (citric acid, succinic acid), and other intermediate products (ethanol and intermediate additives), in order to produce biopolymers by means of microbes.^{31,32,33} Exploitation of biological techniques in waste management is becoming an important method in feasible improvement.^{32,34}

Solid state fermentation (SSF) is an elective approach, for converting agro-industrial squanders and its byproducts into value-added items, i.e., bioactive substances, bioplastics, and biofuels,³⁵ because it mimics the natural environment. Generally, SSF utilizes waste generated from the agriculture as a medium of low or no financial esteem; instead, their transfer is natural concern. Raw material availability and its cost are the two

significant limitations need to be well thought-out while selecting a raw material in SSF.³⁶ The selected raw material should support the utmost growth of microorganism and as well as high product yield. In the present study, various agro-industrial wastes including rice husk, castor husk, sawdust, sugarcane bagasse and groundnut husk were used for the solid state fermentation. All the five were found to be good substrates as the alpha amylase, glucoamylase and protein content activity was observed.

Maximum yield of α -amylase on all solid substrates except castor husk and groundnut waste was registered on second day of incubation. Higher yield of α -amylase (1.614 U/g of substrate & 0.958 U/g of substrate) was showed on 2nd day on rice husk by *A. protuberus* and *A. unguis*, whereas less yield of α -amylase (0.511 U/g of substrate) was noted on 2nd day when sawdust used as a substrate by *A. protuberus*. Respectively, groundnut husk was showed less yield of α -amylase activity (0.580 U/g of substrate) on 5th day. All solid substrates were showed maximum α -amylase activity on 2nd day of incubation except groundnut fodder by *A. unguis* (Fig.3 &5)

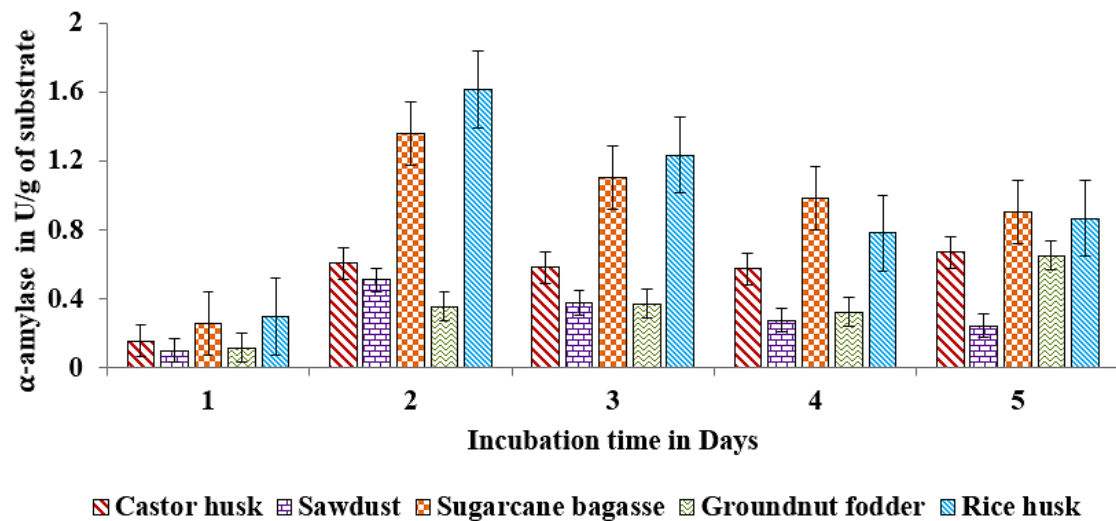


Fig. 3: α -Amylase production by *Aspergillus protuberus* in SSF

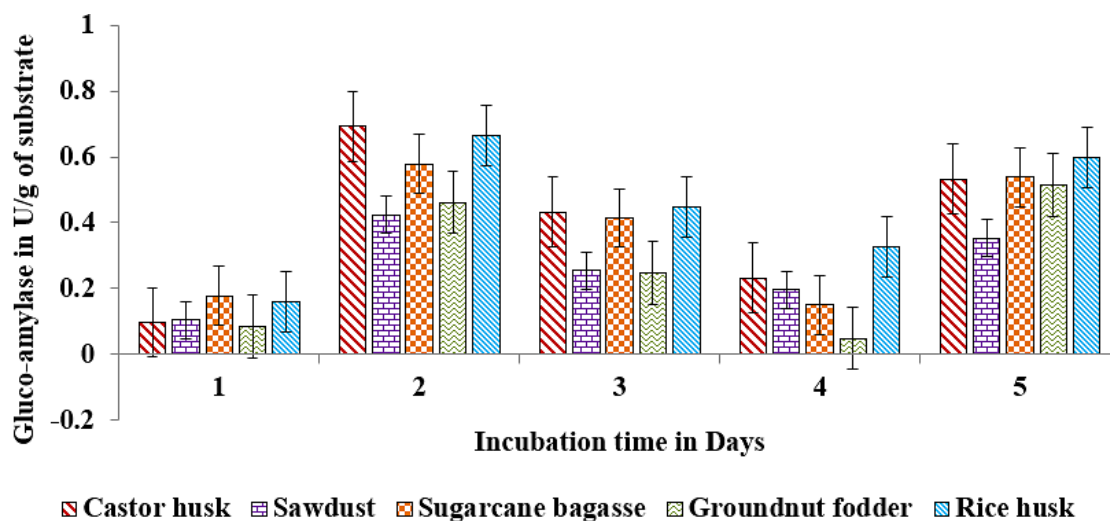


Fig. 4: Gluco- amylase production by *Aspergillus protuberus* in SSF

Maximum titres of glucoamylase on all solid substrates like sugarcane bagasse, rice husk, castor husk and sawdust was showed on the second day of incubation with *A. protuberus*. Among solid substrates tested, castor husk yielded highest activity of 0.692 U/g of solid substrate as against 0.665 U/g of solid substrate by rice husk on 2nd day of incubation. Growth of *A. protuberus* lowest activity of glucoamylase on groundnut husk on 5th day of incubation. By using *A. unguis* on production of glucoamylase was high in sugarcane bagasse, rice husk and groundnut fodder on 1st day of incubation, similarly remaining two substrates showed on 5 h day of incubation. Groundnut waste was observed highest production of glucoamylase (0.847 U/g of substrate) on 1st day followed by rice husk yield of 0.828 U/g of substrate and very less activity of glucoamylase (0.455 U/g of substrate) on 5th day (Fig.4 & 6).

Among the solid substrates used in the present study, maximum secretion of extracellular protein (2.259 and 1.898 mg/g of substrate) on castor husk at peak time interval on 2nd day and 5th day of incubation was recorded in *A. protuberus* and *A. unguis*. Whereas the protein content was low (0.253 and 0.245 mg/g of solid substrate and) on rice husk at peak time interval on 3rd & 1st day of incubation by *A. protuberus* and *A. unguis* (Fig.7 & 8).

Bindu naik *et.al.*³⁷ screened various agro-waste for the production of pullulanase enzyme from the endophytic *Aspergillus* sp. and found that wheat bran was found to be the best substrate with maximum yields of 65.33± 2.08U/gds. Shruthi *et.al.*,³⁸ screened locally available many agro industrial waste material and observed the considerable production of FPase (5.9 FPU/g of substrate), CMCCase (1.1 U/g of substrate) and β -glucosidase activity (6.5 U/g of substrate) and extracellular protein (27.0 mg/g of substrate) in SSF using ground nut fodder. Three different solid wastes viz., sugarcane bagasse, sawdust, and tea residue was screened in SSF and demonstrated that sugarcane bagasse was the excellent solid support among the diverse solid substrates used in this study for higher generation of cellulase and amylase. Sugarcane bagasse backed surprising generation of FPase (15.42 FPU/g of substrate), CMCCase (17.89 U/g of substrate) and β -glucosidase (2.43 U/g of substrate), and amylase activity (15.12 U/g of substrate) in SSF. Noteworthy secretion of protein (45.75 mg/g of substrate) on sugarcane bagasse was taken note.³⁹ In spite of the fact that, production of ethanol from lignocellulosic substrates in a cost effective way, it is a vital challenge with the commercialization of the production practice.

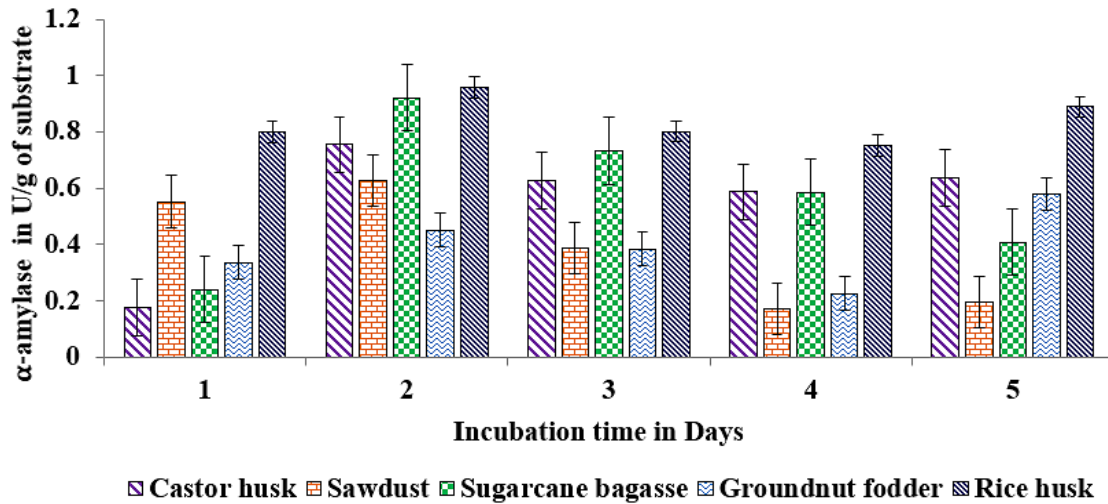


Fig. 5.: α -Amylase production by *Aspergillus unguis* inSSF

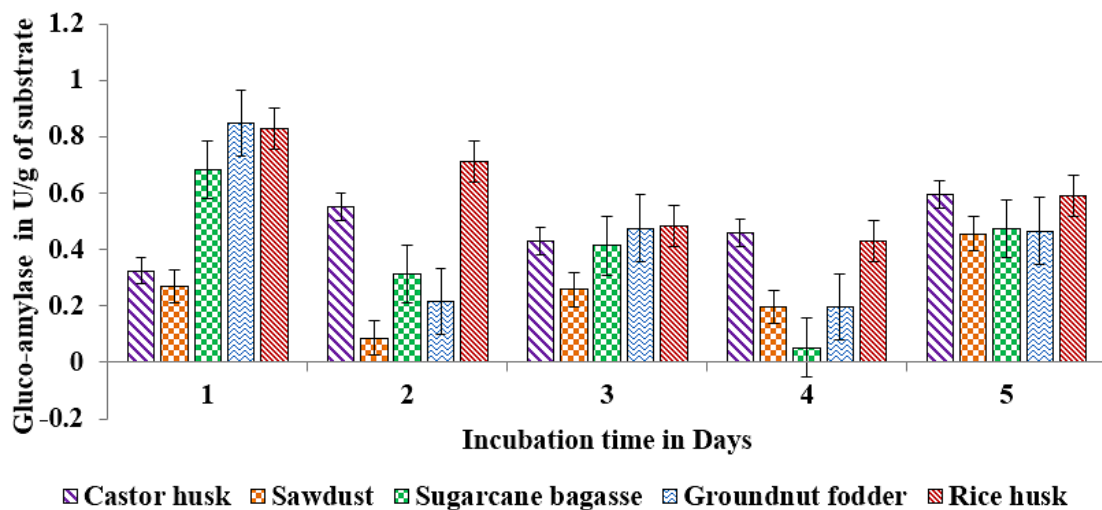


Fig. 6: Gluco- amylase production by *Aspergillus unguis* inSSF

Co-culture Method

Co-cultured microorganisms have advantageous over the individual cultures as the synergistic action of metabolic way of organisms which naturally upgrade the production of commercial enzymes. In nature organisms are not survived as pure cultures and also microbes belong to identical genus encompass the better compatibility with each other, hence, widely used to produce different metabolites. This can be concluded from reports on pair of microbes such as *Bacillus cereus* and *B. thuringiensis*, *Aspergillus niger* MS23, and *A. terreus* MS105, *Clostridium thermocellum* ATCC

27405 and *C.beijerinckii* ATCC 51743, *Aspergillus flavus* and *A. penicillioides*.^{40,41,42,43} Maximum levels of α -amylase (12 u/g of substrate) was record on 2nd day of fermentation when inoculated both cultures at 1:1 ratio and the enzyme secretions are gradually decrease when the incubation period is increasing from 2nd day to 10th day (Fig. 9). Dreaded amount of α -amylase was observed when inoculated these cultures at 1:3 ratio on 10th day of incubation. Maximum levels of gluco-amylase (3 u/g of substrate) was observed on 2nd day of fermentation when inoculated both cultures at 0.5: 0.5 and the enzyme secretions are gradually decrease when the incubation period is

increasing from 2nd day to 10th day (Fig. 10). Dreaded amount of gluco-amylase was observed when we inoculated these cultures at 0.5:0.5 ratio at 10th day of incubation.

concentration of extra cellular protein (20 mg/g of substrate) on rice husk on 2nd day of incubation. Whereas the 0.5:0.5 combination was the poor combination to produce extracellular protein content (2.5 mg/g of solid substrate) on rice husk at peak time interval on 10th day of incubation (Fig.11).

Among the combinations used in the present study, 1:3 combination was good for the production of highest

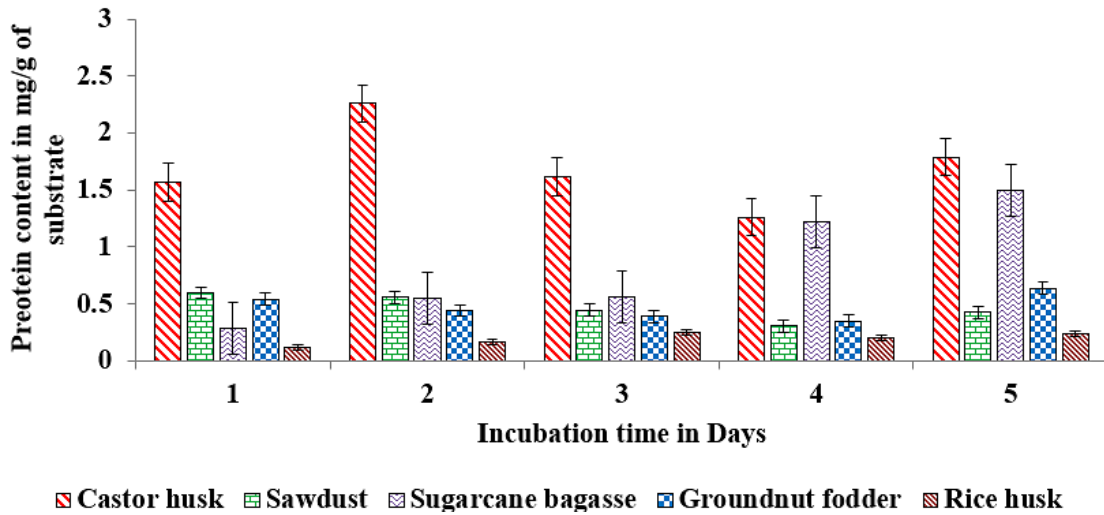


Fig. 7: Production of extracellular protein by *A. protuberus* inSSF

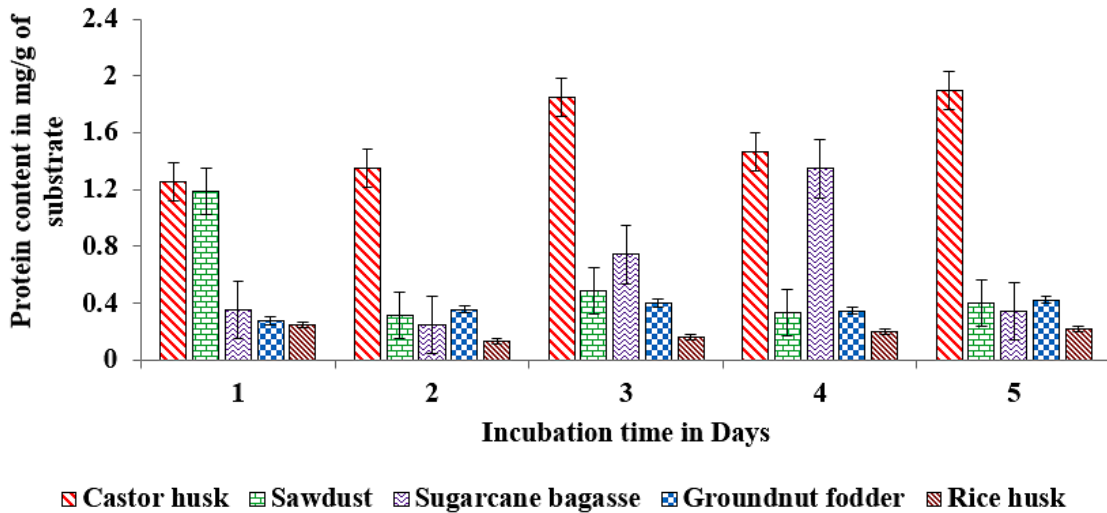


Fig. 8: Production of extracellular protein by *Aspergillus unguis* inSSF

In co-culture study Ankit Iodha *et al.*,⁴⁴ observed the elevated levels of cellulase enzyme (6.71 FPU/gds) when steam pretreated wheat bran was supplemented with 10⁶ spores of each of the *Trichoderma reesei* and *Penicillium citrinum* in SSF at 30 °C, pH 5 and 70% moisture content on 6th day of incubation. In another co-culture

study *Saccharomyces cerevisiae* Y3401 and *Wickerhamomyces anomalus* Y3604 was employed for the utmost production of ethyl acetate i.e., 6.4 g/l at the ratio of 3:1 than in individual fermentation.⁴⁵ Ledys *et al.*,⁴⁶ used triple co-culture study for the enhanced production of ligninolytic enzymes and to decolorize Reactive black dye and found that the

inoculation of 1000 μ L of each culture of *T. viride* and *A. terreus* into the 7-day culture of *Leptosphaerulina*

sp is the best combination than monoculture and previously used chemical inducers.

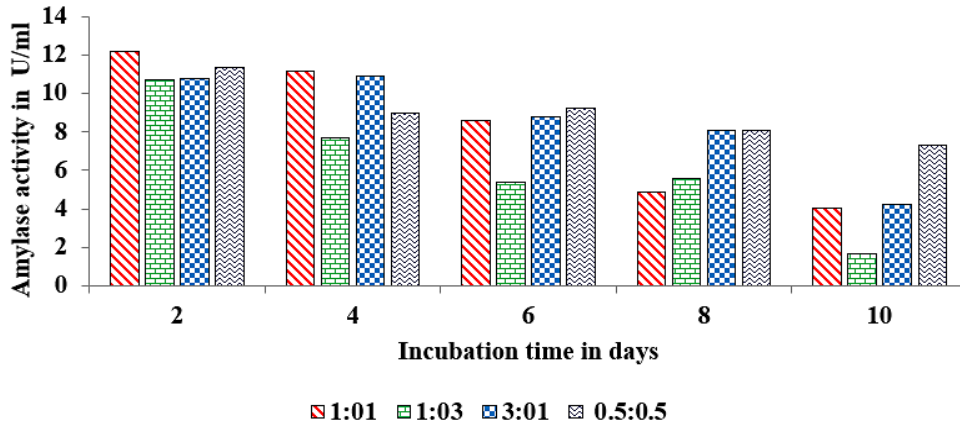


Fig. 9: α -Amylase production by co-culture in different spore ratio in SmF

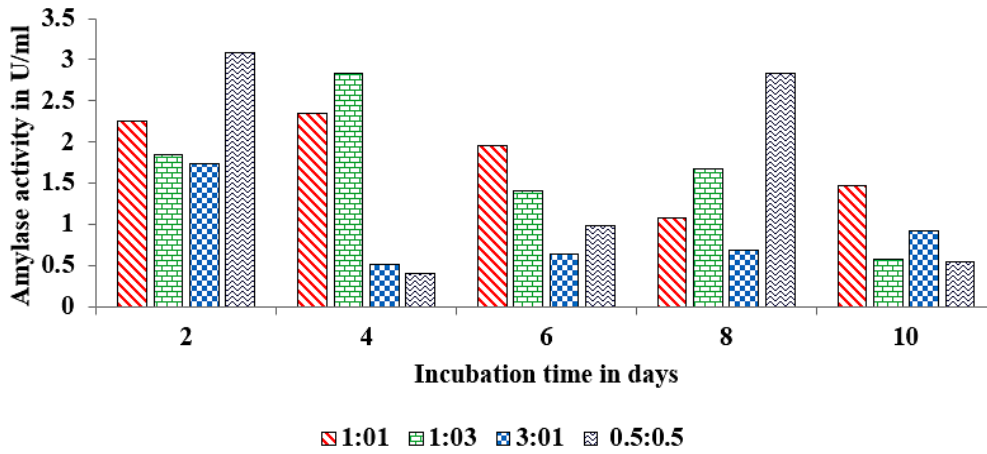


Fig. 10: Gluco- amylase production by co-culture in different spore ratio in SmF

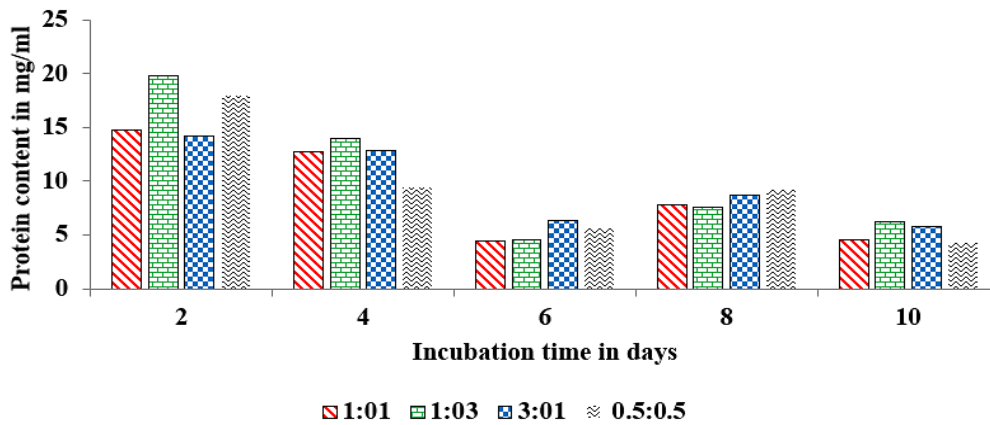


Fig. 11: Production of extracellular protein by co-culture in different spore ratio in SmF

Conclusions

Production of low cost amylase enzyme is the main goal of the present study. Hence various agricultural residues were studied to carry out SSF by *Aspergillus* sp and demonstrated that the studied *Aspergillus* strains has the potential to utilize agricultural waste residues for the production of α -amylase, glucoamylase enzyme and protein content individually. The enzyme productions and extra cellular protein was drastically increased in co-culture study. Hence, concluded that co-culture method is the best method for the enhanced production of amylase enzyme in SmF on rice husk by *Aspergillus* strains in low cost manner.

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Conflict of Interest

The authors have no financial or personal conflict of interest.

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