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Economic Co-Production of Cellulase and α-Amylase by Fungi Grown on Agro-Industrial Wastes Using Solid-State Fermentation Conditions

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ABSTRACT

Eleven fungal cultures belonging to *Trichoderma*, *Penicillium* and *Aspergillus* were grown on corn flour as main substrate of solid-state fermentation "SSF". Highest productions of α-amylase by *T. koningii*, *A. fumigatus* strain KF993 and *Penicillium* sp. F1 were 43.5, 38.5 and 37.8 U, respectively**.** Whereas, maximum productions of cellulase (as filter-paperase activity) were attained by *A. niger* F93 (96.5 U), *A. fumigatus* KF256 (80.1 U), *T. koningii* (86.1 U), *T. reesei* (84.5 U), and *Penicillium* sp. F2 (76.9 U)**.** Hence, in general, *Aspergillus* & *Trichoderma* species produced more cellulase and α-amylase enzymes than did *Penicillium* spp. On the other hand, *Aspergillus* spp. produced, in general, higher amounts of cellulase, but lower amounts of α-amylase, than those of *Trichoderma***.** Supplementing the SSF substrate corn flour with sugarcane molasses has initiated the production of cellulase by five fungal cultures of *A. oryzae* (~245%), *T. koningii* (~7%), *T. viride* F416 (~20%), *Penicillium* sp. F1 and F2 (~248 & 3%, respectively). Also, supplementation with molasses has initiated the production of α-amylase by *A. oryzae* (~13%)**.** Thermophilic *A. fumigatus* KF993 was grown on different agro-industrial residues as main SSF substrates. It produced different amounts of cellulase as filter-paperase activity when grown on corn flour (55.8 U), fodder yeast (52.2 U), fine wheat bran (48.4 U), sugar beet pulp (42.7 U), olive cake (22.7 U), coarse wheat bran (11.7 U), and potato starch (1.7 U). On the other hand, the fungus produced different amounts of α-amylase when grown on corn flour (36.4 U), sugar beet pulp (34.8 U), coarse wheat bran (29.9 U), potato starch (18.2 U), fodder yeast (16.5 U), olive cake (11.6 U), and fine wheat bran (4.1 U)**.** So, using corn flour as SSF substrate showed the highest co-production of both cellulase and α-amylase from thermophilic *A. fumigatus* KF993**.** In addition, the effect of particle size of SSF agro-industrial residue substrate (wheat bran) on cellulase (a filter-paperas activity) and α-amylase productions by thermophilic *A. fumigatus* KF993 was tested. Applying fine particles of wheat bran has increased the production of filter-paperase (up to 4x); in contrast, the coarse wheat bran initiated the production of α-amylase (up to 7x)**.** Thermophilic *A. awamori* AF727 was grown, for 72 h, on different agro-industrial residues as main SSF substrates. It produced different amounts of cellulase as filter-paperase activity; 76.9 U (from sugar beet pulp), 64.7 U (from corn flour), 59.8 U (from olive cake), and 52.6 U (from fodder yeast)**.** On the other hand, it produced different quantities of α-amylase, 37.3 U (from sugar beet pulp), 33 U (from corn flour), 32.1 U (from fodder yeast), and 1.9 U (from olive cake)**.** Thus, applying sugar beet pulp as SSF substrate resulted in the highest co-production of both cellulase and α-amylase from thermophilic *Aspergillus awamori* AF727**.** On the other hand, most *A. awamori* AF727 SSF-cultures has attained their highest production of α-amylase after 24 hours. As for cellulase production, most of *A. awamori* cultures reached their maximum production of cellulase after 48 h (grown on corn flour) or 72 h (grown on olive cake or sugar beet pulp).

Key words α-Amylase, Cellulase, Fungi, Solid-state fermentation (SSF), Economic production, Cost-effective substrates, Agro-industrial residues

Introduction

With global population predicted to hit 9 billion people by 2050, the need for additional requirements of agriculture and food will arise throughout the globe (UN Press Release 2008). Also, the enormous increase in world population has already resulted in generation of million tons of agricultural wastes (Saxena & Singh 2014). Biotechnological process for production of green chemicals, namely enzymes provides the best utilization of these otherwise unutilized wastes. These agricultural wastes constitute a large source of biomass and have potentially detrimental effects both on the environment and human health if not handled and managed properly. Biotechnology offers the best utilization of this waste as alternative substrates in bioprocesses for the production of products as enzymes and food/feed materials using biological entities like microorganisms (Manpreet *et al*., 2005). Microbial enzymes have wide applications in all industrial to household sector, biotechnological, medicinal and basic research fields and hold the major share in the global enzyme market (Zheng *et al*., 2009). Production of multi enzymes from a single fermentation process helps in reducing the cost

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of the overall production when it comes to industrial application of the enzymes. For efficient and simultaneous production of multi-enzymes in a single fermentation, bioprocesses with a well-established bioengineering are needed to be developed.

Thus, the time has come to explore and utilize the non conventional feeds, even available in small quantities, should be utilized region-wise so that can meet the requirement of feeds for livestock and reduces the competition between human and livestock for common food grains (Prasad & Rao 2013). Judicial use of land and water resources is the need of the hour. The ground portion in plantation crops could be used for different suitable cultivated legume and non legume fodders like shade tolerance, etc., according to the age/growth of the main crop.

To produce enzymes commercially using local resources, solid state fermentation (SSF), an alternative for submerged fermentation for enzyme production, was found to be more favorable; since it can be performed under limited financial and labor requirements (Lee *et al*., 2011). Thus, agro-industrial residuals have potentially been used as substrates of SSF not only for enzymes production but also for other secondary metabolites. In this aspect, countries like Egypt with abundant agro-industrial residuals will be of great advantage. In addition, many research activities focused on SSF, which had lead to a wide range of applications not only at laboratory scale. (Gupte & Madamwar 1997, Gutierrez-Correa & Tengerdy 1998, Hang & Woodams 1998, Kotwal *et al*., 1998, and Sekar & Balaraman 1998) but also at pilot and industrial scale (Durand & Chereau 1988, Xue *et al*., 1992, Durand *et al*., 1996 and Fernández *et al*., 1996)**.** An advantage of SSF over SmF is the inhibition of catabolic repression (from the product formed) by regulation of end product synthesis. Hence, inhibition of enzyme by product is overruled in SSF (Ramesh & Lonsane 1991)**.** In addition, Enzyme production is one of the most important applications of SSF. SSF has advantages over submerged fermentation such as high volumetric productivity, low cost of equipment involved, better yield of product, lesser waste generation and lesser time consuming processes etc. The type of strain, culture conditions, nature of the substrate and availability of nutrients are the other important factors affecting yield of enzyme production (Pandey *et al*., 2001). It is crucial to provide optimized water content and control the water activity for good enzyme production. Agro-industrial substrates are considered best for enzyme production in SSF. The cost of enzyme production by submerged fermentation is higher compared to SSF. They have also proved this by comparing cellulase production costs in SSF and SmF (Tengerdy 1998).

Cellulase is an enzyme complex used for the conversion of lignocellulosic residues and used for production of ethanol, single-cell protein, bleaching of pulp, for treatment of waste papers and for fruit juice extraction. In SSF, using lignocellulosic wastes as substrates can reduce the cost of cellulase production (Xia & Cen 1999). Lignocellulosic materials are cheaper and pretreatment is required to improve their utilization. Pretreatment of lignocellulosic matrix increases the potential of cellulases to act on cellulose, hemicelluloses.

Solid-state fermentation (SSF) is defined as the fermentation process in which microorganisms grow on solid materials without the presence of free liquid (Cannel & Moo-Young 1980). The concept of using solid substrates is probably the oldest method used by man to make microorganisms work for him. In recent years, SSF has shown much promise in development of several bioprocesses and products. Solid-state offers greatest possibilities when fungi are used. Unlike other microorganisms, fungi typically grow in nature on solid substrates such as pieces of wood, seeds, stems, roots and dried parts of animals such as skin, bones and fecal matter i.e. low in moisture. In SSF, the moisture necessary for microbial growth exists in an absorbed state or in complex with solid matrix. However, SSF differs from solid substrate fermentation. In solid substrate fermentation, the substrate itself acts as a carbon source and occurs in absence or near absence of free water. However, in solid-state fermentation, the process occurs in absence or near absence of free water by employing a natural substrate or inert substrate as solid support (Kumar *et al*., 2003). The aim of SSF is to bring cultivated fungi or bacteria in tight contact with the insoluble substrate and to achieve the highest nutrient concentration from the substrate for fermentation. This technology so far is run only on a small scale, but has an advantage over submerged fermentation. Two types of SSF systems have been distinguished depending on the type of solid phase used. The most commonly used system involves cultivation on a natural material and less frequently on an inert support impregnated with liquid medium (Ooijkaas *et al*., 2000). SSF can also be classified based on whether the seed culture for fermentation is pure or mixed. In pure culture SSF, individual strains are used for substrate utilization and with mixed culture; different microorganisms are utilized for the bioconversion of agro-industrial residues simultaneously**.** There is a continuous development in SSF technology over the last three decades (Bhargav *et al*., 2008). The advantages of SSF processes overweigh the obstacles due to engineering problems involved in fermentation processes. Presently, in most SSF systems fungi are more suitable than bacterial strains and yeasts.

Substrates such as agro-industrial residues are proved by many researchers to be better for filamentous fungi. The morphology of filamentous fungi supports them to penetrate the hardest surface due to the presence of turgid pressure at the tip of their mycelium. Hence, the raw materials considered as waste are used for production of value added fine products and reducing pollution problems (Raimbault 1998).

The present study was carried out to investigate the co-releasing of some extra-cellular hydrolytic enzymes (cellulase and α-amylase) by different available fungal SSF cultures, grown on some bio-wastes such as agro-industrial by-products, as main SSF substrates, to be fermented and to modify their chemical composition and digestibility to be more suitable for different applications. On the other hand, enzymes produced themselves have different other applications.

Material and Methods

Fungal Cultures

Eleven strains; 4 strains belonging to 3 species of *Trichoderma*, 2 strains belonging to *Penicillium* spp., and 5 strains belonging to 4 species of *Aspergillus*. All fungal cultures were obtained from Microbial Chem. Dep., Nat. Res. Center, Giza, Egypt.

Substrates

Potato starchy wastes was obtained from Misr Trade Company, Chipsy Factory, Industrial zone, October 6th City, Giza Governorate, Egypt. Sugar beet pulp, the secondary by-product of sugar industry from sugar beet, was obtained from El-Fayoum sugar Factory-El-Fayoum, Egypt. Sugarcane molasses and fodder yeast were obtained from Egyptian company for Sugar and Integrated Industries, distillation Factory, Hawamdya, Giza, Egypt. Corn flour and wheat bran were from local markets. Olive cake was from olive pressing Factory, South Sinai Governorate. Ten grams of each substrate was added for each flask (250 ml).

Inoculum Preparation

For experimentation purposes, fungal spore suspensions were prepared by incubating the cultures on malt extract agar slants at 30°C for 7 days. The spores were harvested by using 15 ml sterile water and 1ml of the spores' suspension (10^6/ml) was used for inoculation.

Experimental Culture Conditions

Ten grams of each bio-waste was added in conical flask (250 ml) and incubated at 30°C for the experimental fermentation time. When corn flour was supplemented with sugarcane molasses, the last was added at a concentration of 10% of the corn flour.

Enzyme Activity Analyses

Filter-paperase and α-amylase activities were determined according to the methods of Ghose (1987) and Cordeiro *et al*., (2003), respectively. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar from the appropriate substrates per minute under assay conditions. The enzymatic activities are expressed as units per gram of dry waste (U/g dm).

Determination of Reducing Sugars

Reducing sugars were determined using the method of Miller (1959).

Results and Discussion

In the present study, some factors affecting α-amylase and cellulase productions by different fungal culture strains grown on a number of agro-industrial residues under solid-state fermentation 'SSF' conditions were investigated. Those factors were, mainly, fungal cultures, SSF substrate alone or in combination with sugarcane molasses, different wastes, substrate particle size, and fermentation time.

I. Capability of different Fungal Genera and Species, grown on some agro-industrial residues under solid-state fermentation 'SSF' System Conditions, on Cellulase and α-Amylase Productions

In this study, eleven fungal cultures belonging to *Trichoderma*, *Penicillium* and *Aspergillus* were grown on corn flour as main substrate of SSF (Table 1). Highest productions of α-amylase by different genera and species grown on corn flour were, in descending order, 43.5, 38.5 and 37.8 U by *Trichoderma koningii*, *Aspergillus fumigatus* strain KF993 and *Penicillium* sp. F1, respectively. Thus, maximum production of α-

amylase was attained by genus *Trichoderma* (specie *koningii*)**.** Whereas, maximum productions of cellulase (as filter-paperase activity) by different genera and species grown on corn flour were attained by *A. niger* F93 (96.5 U), *A. fumigatus* KF256 (80.1 U), *T. koningii* (86.1 U), *T. reesei* (84.5 U), and *Penicillium* sp. strain F2 (76.9 U).

Hence, in the present study, in general, *Aspergillus* & *Trichoderma* species have produced cellulase and α-amylase enzymes higher than those of *Penicillium* spp. On the other hand, *Aspergillus* produced, in general, higher amounts of cellulase than those of *Trichoderma* but lower amounts of α-amylase.

Concerning *Aspergillus*, all tested species of *Aspergillus* (*oryzae*, *niger*, *fumigatus* KF993, *fumigatus* KF256 & *awamori*), grown on different SSF substrates, have produced high levels of cellulases (Tables 1, 2 & 3). Moreover, the amount of cellulase produced by *A. niger* (96.5 U) was the highest amount, in comparison with the other organisms investigated (Table 1).

In this respect, Shash (2002) has considered the fungal strain *Aspergillus flavus* S-7 a true cellulolytic fungus, since it had all the enzyme components needed for the degradation of the crystalline cellulosic materials**.** In addition, Lee *et al.* (2011) have evaluated a novel design of solid-state bioreactor, FERMSOSTAT, in cellulase production using *Aspergillus niger* USM AI1 grown on sugarcane bagasse and palm kernel cake at 1:1 (w/w) ratio. Under optimized SSF conditions [of 0.5 kg substrate, 70% (w/w) moisture content, 30°C, aeration at 4 L/h.g fermented substrate for 5min and mixing for 5 min] about 3.4 U/g of Filter paperase activity (FPase) was obtained. Under the same SSF conditions, comparative studies of cellulase production indicated that FPase produced by *A. niger* USM AI1 was about 35.3% higher compared to *Trichoderma reesei* (2.2U/g).

Their result is in accordance with our findings (Table 1). Since, *A. niger* F93 has produced 96.5 U of FPase activity; whereas *A. reesei* produced 84.5 U.

Cellulose, a polysaccharide consisted of linear β-1,4-linked D-glucopyranose chains, requires three classes of enzymes for its degradation: β-1,4-endoglucanases (EGL), exoglucanases/cellobiohydrolases (CBH), and β-glucosidase (BGL). The endoglucanases cleave cellulose chains internally mainly from the amorphous region, releasing units to be degraded by CBHs and/or BGLs. The cellobiohydrolases cleave cellobiose units (the cellulose-derived disaccahride) from the end of the polysaccharide chains (Aro *et al*., 2005). Finally, β-glucosidases hydrolise cellobiose to glucose, the monomeric readily metabolisable carbon source for fungi (Beguin 1990). These three classes of enzymes need to act synergistically and sequentially in order to degrade completely the cellulose matrix. After endo- and exo-cleaving (performed by EGLs and CBHs, respectively), the BGLs degrade the remaining oligosaccharides to glucose. The most efficient cellulose-degrading fungus is *Trichoderma reesei*. The highly efficient degradation of cellulose by *T. reesei* is mainly due to the highly effectiveness of cellulases acting synergistically in this specie, although *T. reesei* does not have the biggest number of cellulases in the fungi kingdom (Ward *et al*., 1993). The *T. reesei* has five characterized EGLs, two highly expressed CBHs and two characterized BGLs, the latter being expressed at low levels (Reczey *et al*., 1998 and Kubicek *et al*., 2011) reviewed in van den Brink & de Vries (2011). In addition to being expressed at very low levels in *T. reesei*, the BGLs are strongly subjected to product inhibition (Chen *et al*., 1992). These features reduce the utilization of *T. reesei* for *in vitro* saccharification of cellulose substrates and, in industrial applications, cellulase mixtures from *T. reesei* are often supplemented with BGLs from Aspergilli, which are highly expressed and tolerant to glucose inhibition (van den Brink & de Vries 2011).

Also, Jadhav *et al*., (2013) found that the growth of *A. niger*, *oryzae* & *flavus* were surplus that of *Penicillium chrysogenum* when grown on 1% CMC containing potato dextrose agar plate for 48 hours.

Hence, their findings are in accordance with the results of the present study (Table 1); since *Aspergillus* species grown on corn as SSF substrate have produced 18.6–96.5 U of FPase; whereas *Penicillium* species have released 18.0 - 76.9 U.

The fungus *Trichoderma reesei* (*Hypocrea jecorina*) is the most important organism used in cellulase production (Stricker *et al*., 2008 a, b) and it has been the focus of cellulases research for over 50 years. Degradation of cellulose is performed by cellulases, a high specific class of enzymes able to degrade the cellulose glycosidic bonds. On the other hand, the filamentous fungi *Aspergillus niger* is known to produce a wide range of hemicellulose-degrading enzymes and it has been used for many industrial applications. Hemicellulose is a complex class of polysaccharides composed by different units of sugars. In order to degrade hemicellulose, the organism should be able to produce a large set of enzymes (hemicellulases), acting in a synergistic way to hydrolyze such complex substrate. Therefore, the ascomycetes *T. reesei* and *A. niger* are considered the most important microorganisms for cellulase/hemicellulase production, and constitute the source of these enzymes for industrial applications, including the production of biofuels from plant biomass**.**

So, in the current study (Table 1), *T. reesei* and *A. niger* F93 have produced highest amounts of cellulase (~85 & 97 U FPase), in comparison with other organisms tested. In this respect, Pal *et al*., (2013) stated that enzyme products for ruminant diets are of fungal (mostly *Trichoderma longibrachiatum, Aspergillus niger* & *A. oryzae*) and bacterial (mostly *Bacillus* spp.) origin.

II. Impact of Supplementation of SSF Substrate with Sugarcane-Molasses, on Cellulase and α-Amylase Productions by Fungal Cultures

Supplementing the SSF substrate corn flour with sugarcane molasses has initiated the production of cellulase by five fungal SSF cultures tested (Table 1). They were *Aspergillus oryzae* (~245%), *Trichoderma koningii* (~7%), *T. viride* F416 (~20%), *Penicillium* spp. F1 & F2 (~248 & 3%, respectively). Also, supplementation with molasses has initiated the production of α-amylase by *Aspergillus oryzae* (~13%).

Jash *et al.* (2013) clarified that Sugarcane molasses is a concentrated liquid extract that is a major by-product of sugarcane refining industry. It is a well-known source of energy and a widely available concentrated form of `fermentable carbohydrate' that has no role in human nutrition. Urea is a product, which after hydrolysis into ammonia in the rumen can be used as a nitrogen source by the microbes. Therefore, a supplement containing these two can stimulate the development of microbes in the rumen, permitting a better digestion of the forages and a greater production of microbial protein, which could provide essential nutrients in the intestine. For these reasons, liquid supplements containing molasses and urea have been used in many countries such as Australia, India and those of Southern Africa, for drought feeding and for intensive cattle fattening. The molasses being a concentrated plant juice will provide a range of trace minerals (except for phosphorous) and a complete mixture of vitamins**.** Whereas, cereal brans are high in phosphorus, trace minerals and also a range of vitamins. In addition, they provide a slow-release amino acid source from the relatively insoluble proteins to the microbes. Also, oilseed meals provide both soluble and insoluble proteins and are a good source of phosphorous. It is appropriate to add such ingredients when blocks are given to animals in production.

Table 1. α-Amylase and filter-paperase 'FPase' activities of some fungi cultures, grown on Corn flour at 30°C for 7 days as main substrate of solid-state fermentation (SSF), or supplemented with sugarcane-molasses.

Microorganism		SSF Substrate	Enzyme activity (U; mg/g Substrate)	
			α-Amylase	Filter-Paperase
Trichoderma	*Reesei*	Corn flour	20.1	84.5
		Corn flour + Molasses	18.1	65.4
	Koningii	Corn flour	43.5	86.1
		Corn flour + Molasses	20.8	91.9
	Viride F416	Corn flour	20.4	78.7
		Corn flour + Molasses	17.1	94.1
	Viride s50	Corn flour	18.2	78.7
Penicillium	Sp. F1	Corn flour	37.8	18.0
		Corn flour + Molasses	20.0	69.2
	Sp. F2	Corn flour	20.3	76.9
		Corn flour + Molasses	19.7	78.9
Aspergillus	*Oryzae*	Corn flour	20.4	18.6
		Corn flour + Molasses	23.0	64.1
	niger F93	Corn flour	20.2	96.5
	fumigatus KF993	Corn flour	38.5	40.8
		Corn flour + Molasses	14.5	19.7
	fumigatus KF256	Corn flour	18.7	80.1
	awamori AF727	Corn flour	28.7	47.9

III. Outcome of applying some Agro-Industrial Residues as Main SSF Substrates on Cellulase and α-Amylase Productions by thermophilic Aspergillus fumigatus

Thermophilic *Aspergillus fumigatus* KF993 was grown on different agro-industrial residues as main SSF substrates (Table 2). It produced different amounts of cellulase as filter-paperase activity when grown on, in descending order, corn flour (55.8 U), fodder yeast (52.2 U), fine wheat bran (48.4 U), sugar beet pulp (42.7 U), olive cake (22.7 U), coarse wheat bran (11.7 U), and potato starch (1.7 U). On the other hand, it produced also different amounts of α-amylase when grown on corn flour (36.4 U), sugar beet pulp (34.8 U), coarse wheat bran (29.9 U), potato starch (18.2 U), fodder yeast (16.5 U), olive cake (11.6 U), and fine wheat bran (4.1 U). Thus, using corn flour as SSF substrate resulted in the highest co-production of both cellulase and α-amylase from thermophilic *Aspergillus fumigatus* KF993.

da Silva *et al*. (2013) observed that milled corn and oatmeal together with soluble potato starch, which have high content of ions and vitamins, were important compounds for amylase production and the growth of the *Aspergillus niveus***.** They analyzed the effect of several nutritional and environmental parameters on amylase production by a novel thermo tolerant filamentous fungus *Aspergillus niveus*. That strain produced high levels of amylolytic activity in Khanna liquid medium supplemented with commercial starch, initial pH 6.5, under static conditions for 72 h. Among the tested carbon sources, milled corn, oatmeal, soluble potato starch and Maisena^{TM} (commercial product obtained from corn starch) were the best inducers of enzymatic secretion. Also,

maltose, wheat bran, amylopectin and raffinose also demonstrated an excellent induction of amylases. The main products of hydrolysis analyzed by thin layer chromatography were glucose, maltose and traces of maltooligosaccharides, suggesting the presence of α-amylase and glucoamylase activities in the crude extract. On the other hand, other carbon sources tested such as rice straw, constituted basically of cellulose and hemicellulose, were not specific inducers of amylase. Sugar cane bagasse, corn cob sucrose, lactose and arabinose were the worst inducers of amylase synthesis, showing amylolytic yields close to the filtrate obtained from cultures incubated without carbon sources**.** The presence of three enzymes with amylolytic activity in gel electrophoresis revealed the presence of a complex amylolytic system that efficiently produces glucose, maltose and maltooligosacchacarides as hydrolysis product of starch.

Bhargav *et al.* (2008) reported that wheat bran is the best SSF substrate for α-amylase from fungi. In this respect, Delabona *et al.* (2013) found that *Aspergillus fumigatus* P40M2 grown on wheat bran and soybean bran has produced ~3.5 U of cellulase (as filter paper activity) /g, after 5 days of fermentation period.

Wheat-bran has texture and porosity that facilitate mycelial dispersion (Madruga & Camara 2000 and Pandey 2003)**.** It is the outer ~15% of the wheat seed and is composed predominantly of non-starch carbohydrates (~58%), starch (~19%) and crude protein (~18%), with the non-starch polysaccharides being primarily ~70% arabinoxylans, ~24% cellulose and ~6% β-(1,3) (1,4)-glucan (Carré & Brillouet 1986, Ralet *et al*., 1990, Wayman & Chen 1992 and Maes & Delcour 2002)**.** Wheat bran or acid-hydrolyzed wheat bran can increase cellulase production by filamentous fungi (Wayman & Chen 1992, and Palmarola-Adrados *et al*., 2005). Also, wheat-bran culture gave the richest gene expression profile of hydrolytic enzymes from *Aspergillus oryzae* among the three tested media (Maeda *et al*., 2004). However, which factor in wheat bran being important for cellulase synthesis is unknown yet**.** It is generally believed that oligosaccharides play an important role in regulating the synthesis of wood-degrading enzymes (Hrmova *et al*., 1991 and Schmoll & Kubicek 2003), and oligosaccharides has been proved to be converted to inducer (such as sophorose and gentiobiose) by transglucosylation (Suto & Tomita 2001, Kurasawa *et al*., 1992, Kubicek 1987, and Claeyssens *et al*., 1990). So, Sun *et al*., (2008) supposed that oligosaccharides may exist in autoclaved wheat bran, which may play an important role in increasing the production of the extracellular biomass-hydrolyzing enzymes by *P. decumbens*. The experiments reported by Sun *et al*. (2008) were undertaken to identify the components in wheat bran responsible for the stimulation of growth and the increased production of the industrially important biomass-degrading enzymes by *P. decumbens*. The knowledge obtained may provide a scientific platform for further improvement of the processes that employ this fungus for biomass conversion.

Later, in a study explained the stimulation of wheat-bran, Sun *et al*. (2008) examined the effects of the starch, protein, and soluble oligosaccharides contents in wheat-bran on the extra-cellular biomass-hydrolyzing enzymes activities released by fungal mycelia grown in batch fermentations. Their results showed that increased starch content correlated directly with an increase in released amylase activity but inversely with the levels of secreted cellulase and xylanase. Moreover, they compared the effects of the soluble and insoluble components of wheat bran and cello-oligosaccharides supplements on production of extra-cellular cellulase and xylanase. They revealed that the soluble cello-oligosaccharides compositions in wheat bran were proved to be one of the most significant factors for cellulase production. According to the results of that research, determining and regulating the composition of wheat bran used as a fermentation supplement may allow for improved induction of cellulase and xylanase production.

Sugar beet pulp is the solid byproduct left after sugar extraction from sugar beet, and comprising 6% of the total fresh weight of the harvest sugar beet (Kjaergaard 1984). It contains 26.5% cellulose, 19.7% hemicellulose and pectin 18.6% (Khalel *et al*., (2007); and it is available in the local market and usually used as a feedstuff for ruminants. It has a similar feeding value as that of grains (corn and barley)**.** Hence, cereal grains, as feedstuff for ruminants, are partially replaced by sugar beet pulp, biologically treated with microorganisms to enhance the performance of sheep (Khalel *et al*., 2007). Biological treatments using *Trichoderma reesei* or *viride* or fibrolytic enzymes were applied to improve the nutritive value & digestibility of the poor quality roughages (Yang *et al*., 1999).

Aspergillus sp. and *Trichoderma* sp. were used in different studies to enhance the protein content of Sugar Beet Pulp and to hydrolyze pectin and lingo-cellulolytic bonds, which in turn increase the soluble carbohydrate fraction and decrease the structural carbohydrate, particularly hemicellulose (Blumenthal 2004 and Mohamed 2005).

In the olive oil production process large quantities of waste are generated in a form of olive cake (as a solid residue) and vegetation water (as a liquid residue). From 100 kg of olives, by three-phase processing system, 50 kg of fresh olive cake is obtained (Fokaides & Tsiftes (2007). Utilization of this kind of waste is becoming a larger issue each day. Opening of many little oil mills and olive orchard planting is contributing to that problem. The result is uncontrolled disposal of olive cake in the environment, which has a negative influence not only on the environment, but on the tourism as well; because of the unpleasant odor that derives form this kind of waste. Olive cake consists of fruit skin, pulp and pit fragments, and the main chemical ingredients are cellulose, proteins, poly-phenols and water. Water content varies with the process used for oil

production, thus in olive cake obtained by pressing process is lower than in the one obtained by centrifugation. Chemical composition of olive cake depends on the sort, condition and origin of the olives, as well as on the processing used. Regarding its chemical composition, olive cake can present s valuable raw material which could be used for useful organic components production, energy generation but also as a component in animal feed (Fernández-Bolaños *et al*., 2006).

Wide-scale industrial applications of enzymes require their cost-effective production to make the process economically viable (Dobrev *et al*., 2007). This can be achieved by using cheaply available agro-industrial residues such as wheat bran, oat bran, rice straw, or others (Kapoor *et al*., 2008). Annually, large quantities of lignocellulosic wastes are generated through industrial processes (Anuradha *et al*., 2007). So, this can be utilized for economic production of enzymes by microorganisms through fermentation processes (Milagres *et al*., 2004 and Okafor *et al*., 2007).

Microbial enzymes are preferred to plant enzymes due to their short growth period, higher productivity and thermostability (Mishra & Behera 2008). Amylases have been reported to be produced by a number of fungi, including *Aspergillus*, *Rhizopus*, *Fusarium*, *Candida*, *Penicillium*, *Thermomucor*, basidiomycete Fomitopsis and Thermomyces (Balkan & Ertan *et al*., 2005, Kumar *et al*., 2007, Kunamneni *et al*., 2005, Mohamed *et al*., 2007 and Yoon *et al*., 2006). Majority of the studies on fungal amylases are based on mesophiles, rarely on facultative thermophiles (Maheswari *et al*., 2000). Current researches focus on thermotolerant enzymes from thermophilic microbial strains.

IV. Effect of Particle Size of SSF Agro-Industrial Residue Substrate on Cellulase and α-Amylase Productions by Thermophilic Aspergillus fumigatus KF993

Effect of particle size of SSF agro-industrial residue substrate (wheat bran) on cellulase (a filter-paperas activity) and α-amylase productions by thermophilic *Aspergillus fumigatus* KF993 was investigated (Table 2). Applying fine particles of wheat bran has increased the filter-paperase activity of the organism 314% (up to 4x); while, in contrast, the coarse wheat bran initiated the production of α-amylase 629% (up to 7x). Thus, coarse wheat-bran resulted in production of moderate amount of amylase (~30U); whereas the soft one led to high amount of cellulase (~48U of FPase), in comparison with other substrates, from thermophilic *Aspergillus fumigatus* KF993.

Laudadio *et al.* (2010) stated that wheat middling are a by-product of the wheat milling industry and these by-products have the potential to reduce livestock feeding costs. Middling consist of coarse and fine particles of bran, shorts, germ, flour, and the offal from the tail of the mill.

Bhargav *et al.* (2008) reported that selection of agro-industrial residues for utilization in SSF depends on some physical parameters such as particle size, moisture level, intra-particle spacing and nutrient composition within the substrate.

In addition, Babu & Satyanarayana (1995) cleared that nutrient supplementation from organic sources increases enzyme production to a greater extent than inorganic sources. It has also been found that most researchers used wheat bran as substrate for enzymes production because it contains sufficient nutrients; it remains loose even in moist conditions, and has a larger surface area. Due to these factors, aeration and mycelial penetration are easier in wheat bran.

In the present study, lowering the size of wheat-bran granules inhibited the α-amylase activity and, in contrast, stimulated the cellulase activity (Table 2). This finding is not in accordance with that of Sun *et al*., (2008) who found that at smaller granule sizes of wheat-bran, the net starch content increased and this resulted in increased amylase production by *Penicillium decumbens* but reduced total mycelial growth and lowered levels of cellulase and xylanase released**.** Hence, applying different organisms in both studies may led to these dissimilar results.

Table 2: α-Amylase and filter-paperase activities of thermophilic *Aspergillus fumigatus* KF993 grown at 30°C for 7 days on agro-industrial bio-wastes as main substrates, under SSF conditions.

SSF Substrate	U (mg/g Substrate)	
	α-Amylase	Filter-Paperase
Coarse wheat bran	29.9	11.7
Fine wheat bran	04.1	48.4
Corn flour	36.4	55.8
Potato Starch	18.2	01.7
Olive cake	11.6	22.7
Fodder yeast	16.5	52.2
Sugar beet pulp	34.8	42.7

V. Efficiency of using some Agro-Industrial Residues as Main SSF Substrates for Cellulase and α-Amylase Productions by thermophilic Aspergillus awamori AF727

Thermophilic *Aspergillus awamori* AF727 was grown, for 72 h, on different agro-industrial residues as main SSF substrates (Table 3). It produced different amounts of cellulase as filter-paperase activity; namely in descending order, 76.9 U (from sugar beet pulp), 64.7 U (from corn flour), 59.8 U (from olive cake), and 52.6 U (from fodder yeast). On the other hand, it produced different quantities of α-amylase, namely in downward order, 37.3 U (from sugar beet pulp), 33 U (from corn flour), 32.1 U (from fodder yeast), and 1.9 U (from olive cake).

Thus, applying sugar beet pulp as SSF substrate resulted in the highest co-production of both cellulase and α-amylase from thermophilic *Aspergillus awamori* AF727.

In this respect, Shukla *et al*., (2014) have optimized a consortium of four hypercellulolytic fungal cultures, namely *Aspergillus awamori*, *Trichoderma viride*, *A. nidulans* and *Phanerochaete chrysosporium* for compost production on the basis of their lignocellulolytic enzyme production potential., The consortium has been effectively used for composting of diverse agricultural wastes.

VI. Outcome of Fermentation Time for cellulase and α-Amylase Productions by Aspergillus awamori AF727 grown on some Agro-Industrial Residues as Main SSF Substrates

In the current study, most *Aspergillus awamori* AF727 SSF-cultures has attained their highest production of α-amylase after 24 hours, except for those grown on fodder yeast which attained their production peak later after 48 hours (Table 3). As for cellulase production, Most of *A. awamori* cultures reached their maximum production of cellulase after 48 h (grown on corn flour) or 72 h (grown on olive cake & sugar beet pulp). The late of attaining maximum productions of cellulase could be related to the complex nature of cellulase system.

In this respect, Bhargav *et al*., (2008) stated that some factors like moisture content, particle size, pH, incubation temperature, inoculum size, incubation period and enrichment of medium with carbon and nitrogen were considered optimum for cellulase production.

Shash (2002) cleared that the cellulolytic productivity varied greatly with the variation of fermentation period. Moreover, cellulase enzyme system is relatively complex and the ratio of these enzyme components change with the age of culture (Umezurike 1975).

Table 3. α-Amylase and filter-paperase activities of thermophilic *Aspergillus awamori* AF727 grown at 30°C for different fermentation times on agro-industrial bio-wastes as main substrates, under SSF conditions.

SSF Substrate	Fermentation Time (h)	U (mg/g Substrate)	
		α-Amylase	Filter-Paperase
Fodder yeast	24	24.3	52.6
	48	32.1	43.6
	72	19.6	34.0
Olive cake	24	01.9	17.4
	48	00.0	20.9
	72	00.0	59.8
Sugar beet pulp	24	37.3	56.1
	48	27.2	59.1
	72	28.6	76.9
Corn flour	24	33.0	10.1
	48	21.2	64.7
	72	31.9	10.1

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Conclusion

Thus, in the present study, growing fungi on bio-wastes has activated their releasing of some hydrolytic enzymes hydrolyzing matched substrates in those wastes (Tables 1, 2 & 3). Consequently, these fungi-treated wastes became more suitable for different applications**.**

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