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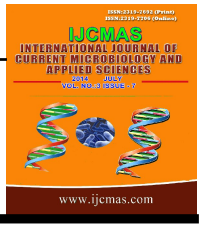

Original Research Article

Improved Xylanase production by mixing low cost wastes and novel co-culture of three marine-derived fungi in solid state fermentation

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Keywords

Agricultural wastes, Co-cultivation, Xylanase, Marine-derived fungi, Solid-state fermentation.

A B S T R A C T

Screening of eighteen marine-derived fungal isolates for xylanase production indicate that, *Aspergillus flavus*, *Cladosporium sphaerospermum* and *Epicoccum purpurascens* were the most promising microorganisms. Solid-state fermentation (SSF) on five agro-industrial wastes showed differential utilization of the various carbon sources by the three isolates. The order of xylanase production was as follows, *A. flavus*> *C. sphaerospermum* > *E. purpurascens*, recording the highest xylanase production on wheat bran (32.40± 1.09, 25.03± 0.85, 18.65± 0.68 U/g, respectively) followed by saw dust. Mixing of wheat bran and saw dust with different ratios improved the enzyme production by 21.82, 50.22 and 22.09 %, respectively. Co-cultivation of the three isolates on wheat bran and saw dust with ratio of (1.5:1.5 w: w), improved xylanase production by 55.41, 63.14 and 156.22%, respectively, compared to mono-cultures. These data demonstrate that, novel co-cultivation of the three marine-derived fungi results in improved production of the biotechnologically relevant enzyme.

Introduction

The existence of pollution problems associated with agro-industrial wastes, scarcity of places for its disposal, costlier treatment options and increased need to save valuable resources have forced to encourage the utilization and bioconversion of waste into high industrially products, bioconversion of the agricultural wastes through microbial fermentation is the natural way to recover resources (Rajoka et al., 2012).

Xylanases (EC 3.2.1.8) are a class of enzymes which degrade the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose, which is a major component of the cell wall of plants. For this reason, importance of microbial xylanases have increased for the production of hydrolysate from agro-industrial wastes, in nutritional improvements of lignocellulosic feeds, processing of food, in increasing animal feed digestibility, biobleaching of paper pulp, clarification of fruit juices, the extraction of plant oil, coffee and starch (Ahmed et al., 2009).

Xylanases have also been studied for the production of xylo-olgiosaccharides, which are used as moisturizing agents for food, sweeteners, and specific health food (Teixeira et al., 2010). Xylanolytic enzymes have also opened new possibilities for the bioconversion of agricultural wastes into easy fermentable sugars (Romdhane et al., 2010).

Filamentous fungi are attracting greater attention than the bacteria and actinomycetes as potential sources of xylanases because they secrete high levels of the enzymes into the culture medium (Berry et al., 1990).

Pre-digestion of the carbon source (e.g. plant-derived polysaccharides) occur extracellularly for fungi, and therefore it is highly likely that natural degradation of plant biomass occurs by mixtures of enzymes produced by several organisms. Co-cultivation of fungi may therefore result in more efficient enzyme mixtures for industrial applications than those obtained from mono-cultivations. Fungal co-cultivations have been previously described for the production of specific enzymes. *A. niger* and *Trichoderma reesei* were co-cultivated for cellulase production (Maheshwari et al., 1994), while *Aspergillus ellipticus* and *Aspergillus fumigatus* were co-cultivated for cellulase and β-glucosidase production (Gupte and Madamwar, 1997).

Increasing of production of xylanase is a prerequisite for its economical manufacturing. In this regard, we focused on improvement of xylanase production by mixing the low cost substrates under SSF. In addition, we performed co-cultivations of the selected three marine-derived fungi, respectively to see whether this could improve enzyme production.

Materials and Methods

Microorganisms and inoculum

The marine-derived fungal isolates used in this study were *Aspergillus flavus*, *Cladosporium sphaerospermum* and *Epicoccum purpurascens*. These isolates were locally isolated from decayed wood samples of old fishing boats collected from Ismailia, Egypt, and grown on malt extract agar (MEA). The medium is composed of biomalt 20g/l, agar15g/l, 800 ml sterile sea water and 200 ml distilled water (Höller et al., 1999). To investigate the growth rate and mycelial interactions, combinations of fungi, plate growth experiments were performed by growing cultures on MEA as follow, *A. flavus + C. sphaerospermum*, *A. flavus + E. purpurascens*, *C. sphaerospermum+ E. purpurascens*, and *A. flavus + C. sphaerospermum+ E. purpurascens.*

For experimentation purposes, spore suspension was prepared by incubating the cultures on MEA slants at 30 ◦C for 7 days, until sufficient sporulation is observed. The spores are harvested using 15 ml steril sea water and 1ml of the suspension was used for inoculation purposes. In co-cultivations, half the number of spores from each species was used equally.

Solid-state fermentation (SSF)

Five different agricultural waste substrates, olive mill waste (OMW), olive leaves (OL), saw dust (SD), wheat bran (WB) and corn cobs (CC) were chosen as substrates for SSF in the present study.

All the wastes obtained locally, air-dried and milled, were screened as fermentation substrates for maximum xylanase enzyme production.

The fermentation was carried out in Erlenmeyer flasks (250 ml) with 3g of the solid substrate moistened with 15 ml of sea water. The flasks were autoclaved for 20 min at 121 ºC and inoculated aseptically with 1ml mycelial suspension. Incubation at 30ºC, under static conditions for 10 days.

Enzyme extraction

After 10 days of the incubation period, the enzyme extraction was done according to Gomes et al (2006), by adding 50 ml of distilled water to the solid cultures and placed on a shaker for 30 min. The suspension was filtered through a nylon cloth, and then centrifuged at 5,000 rpm for 15 min at 4°C. The filtrate obtained was used for determination of xylanase activity.

Primary screening for xylanase production

All fungal isolates were screened for their abilities to produce extracellular xylanase by applying the enzyme extract to medium containing xylan 1% and agar 2%. The inoculated plates were incubated for 24h at 50ºC. Plates were flooded with 0.1% Congo Red and after 30 min, washed with 1 M NaCl and were observed for zone of clearance around the enzyme inoculated hole. Enzyme extracts, which produced distinct clear zone around their holes, were selected (Kulkarni and Gupta, 2013)

Enzyme assay

This was done according to the method of (Warzywoda et al., 1983). 0.5 ml of diluted enzyme solution was added to 0.5 ml of 1% xylan in 0.05 M acetate buffer (pH 5.0). Incubation of the reaction mixture was performed for 30 min at 50˚C. The amount of reducing sugar liberated was quantified by the method of Neish (1952) using xylose as standard.

One unit of xylanase is defined as the amount of enzyme that liberates 1 µmol of xylose equivalents per minute under assay conditions.

All the enzyme activity values presented in graphs and tables are the mean ± standard deviation of three replicates calculated using MS Excel.

Xylanase production on a mixture of wheat bran and saw dust

A mixture of different proportions of WB and SD was studied as next step, after selection of the most favorable substrates for xylanase production.

Three grams of WB and SD in different proportions (2:1, 1.5:1.5, 1:2) along with controls (3:0 and 0:3) were studied to determine the best ratio for xylanase production.

Co-cultivation of fungi

To determine the influence of fungal co-cultivation on xylanase production, the tested fungal species were grown alone (control) or as mixed culture for 10 days in SSF of different WB and SD proportions.

Mixed culture combinations performed by preparing the spore suspension from *A. flavus* and *C. sphaerospermum*, *A. flavus* and *E. purpurascens*, *C. sphaerospermum* and *E. purpurascens* in the ratio of 1:1. Also the mixed culture of the three isolates was studied.

Time course for xylanase production.

In a separate experiment, the time course of xylanase production was studied. The inoculated flasks were incubated for 4, 7, 10 and 14 days.

Results and Discussion

Screening of marine-derived fungal isolates for xylanase production

Eighteen marine-derived fungal isolates were screened for their ability to produce extracellular xylanase after growth on wheat bran as solid state fermentation medium for 10 days. The fungal enzyme extract was tested for xylanase activity on xylan agar medium. The screening revealed that only three were positive for extracellular xylanase production (photo 1). As shown in photo (1) the diameter of clear zone differed depending on the enzyme activity of each isolate. i.e. as the enzyme activity was higher as the diameter of the clear zone was greater. The three isolates were identified as *Aspergillus flavus*, *Cladosporium sphaerospermum* and *Epicoccum purpurascens.* As observed in photo (1) the biggest clear zone was observed in the following order *A. flavus* > *C. sphaerospermum* > *E.purpurascens*.

Ribeiro et al (2014) indicated that, out of different filamentous fungi screened for xylanase production *Aspergillus clavatus* presented the biggest halos at all the temperatures tested. However, Kulkarni and Gupta (2013) reported that *Aspergillus niger* (AS012), *Gliocledium sp* (GS005) and *Trichoderma viride* (TS007) were the best producers for xylanase, showing the biggest clear zones around growing colony, when 32 fungal isolates from soil of social forest area of Bhilai Township were screened on xylan agar plates.

Xylanase production using different agro-industrial residues

It is a well-established fact that culture conditions such as the type of carbon source and time course significantly affect the production of xylanases (Ahmed et al., 2009; Sorgatto et al., 2012). Thus carbon source plays an important role in enzyme production; the choice of an appropriate substrate is of great importance for the successful production of xylanases. The substrate not only serves as a carbon source but also produces the necessary inducing compounds for the organism (Haltrich et al., 1996). The use of agricultural residues as alternative carbon sources reduces the production costs and the price of the final product (Betini et al., 2009).

Xylanase production by *Aspergillus flavus*, *Cladosporium sphaerospermum* and *Epicoccum purpurascens* was evaluated using different natural alternative carbon sources including; olive mill waste (OMW), olive leaves (OL), saw dust (SD), wheat bran (WB) and corn cobs (CC). Fig. (1) Showed differential utilization of the various carbon sources by the three isolates. This result may be attributed to the value of xylan or cellulose: xylan ratios in each substrate (Haltrich et al., 1994; Ghanem et al., 2000).

The highest xylanase production by *A. flavus, C. sphaerospermum* and *E.purpurascens* (32.40±1.1, 25.03±0.85 and 18.65±0.68 U/ g, respectively) was obtained on using the wheat bran as the carbon source followed by saw dust followed by corn cobs, olive mill waste and olive leaves at the last.

The widespread suitability of wheat bran may be due to the presence of sufficient nutrients and its ability to remain loose even in moist conditions thus providing a large surface area (Sivaramakrishnan et al., 2007). The inducing effect of WB is also due to the fact that the cell-wall polysaccharides of WB contained 40% xylans, which were the substrates for xylanase (Sanghi et al., 2008). In addition to xylan, WB also contained about 28% protein which might serve as the

source of carbon and nitrogen for the microorganisms (Dhillon et al., 2011).

Several fungi also produced xylanase from wheat bran such as *Asperillus terreus* and *A.niger* (Gawande and Kamat, 1999), *Thermomyces lanuginosus* (Gaffney et al., 2009), *A.terricola* Marchal (Michelin et al., 2010), *A. foetidus* MTCC 4898 (Chapla et al., 2010). Also, Kar et al (2013) and Guimarãs et al (3013) found that wheat bran was the best substrate for xylanase production by *Trichoderma reesei* SAF3 and *A.japonicus var aculeatus*, respectively under SSF.

It should be noticed that the highest xylanase activity was obtained in the following order *A. flavus* > *C. sphaerospermum* > *E.purpurascens* (32.40±1.1, 25.03±0.85 and 18.65±0.68 U/gm, respectively). These values were higher than that produced by some fungi: e.g. *Paecilomyces themophila J 18* (18.58 U/g of wheat straw) Yang et al (2005), *Thermomyces languginosus D2W3* (11.85 U/g of wheat straw) Sonia et al (2005) and higher than that produced by *A.niger* and *T.viride* (12.5 ±0.13, 11.0 ±0.13 U/g of barley bran) (Soliman et al., 2012)

One of the most important observations that saw dust was also a good inducer for xylanase production by *A. flavus, C. sphaerospermum* and *E. purpurascens*) (24.14 ±1.18, 10.48± 0.45 and 15.32 ±1.17 U/gm, respectively, and this coincides with that found by Murugan et al (2011) and Buthelezi et al (2011) for xylanase production by *Arthrobacter sp.* MTCC 6915 and *Bacillus* strains, respectively. Also, Kumar & Ojha (2013) reported xylanase production by *Trichoderma sp.* on saw dust.

The influence of WB and SD mixture on xylanase production

As shown in the previous experiment, xylanase production was dependent upon the nature of the carbon source used in the culture media. i.e. the use of WB and SD each alone in the fermentation medium induced the highest xylanase activity by the three isolates. So in the following experiment we studied the effect of WB and SD mixing with different ratios (3:0, 0:3, 1.5:1.5, 2:1, 1:2) on xylanase production by our isolates in comparison with using each separately. As shown in fig. (2) the use of WB and SD mixture affected the xylanase production with different degrees on the three isolates, i.e. for *A.flavus* the use of WB and SD mixture with 2:1 ratio increased the xylanase activity (39.47±0.39 U/g) by 21.82% and 63.50% if compared to the use of WB and SD each alone respectively in the fermentation medium. While for *C. sphaerospermum* the use of WB and SD mixture with 1:2 ratio induced the highest xylanase activity (37.60±0.43 U/g) causing an increase by 50.22% and 258.78% as compared to the use of each WB or SD alone, respectively. On the other hand the use of WB and SD mixture with 1.5:1.5 ratio enhanced the xylanase activity (23.94±0.79 U/g) by *E. purpurascens* causing an increase of 22.09% and 56.27% in xylanase activity if compared to the use of WB or SD alone respectively. Some authors used mixtures of other agricultural wastes. e.g. Soliman et al (2012) found that the use of rice straw and wheat bran mixture with 3:1 ratio increased xylanase production by *A. niger* by 168.42% and 410 % in comparison to the use of each agricultural waste alone while the use of the same mixture for xylanase production by *T.viride* did not affect the xylanase production.

In this connection, some literatures proved that the use of agricultural wastes mixture during SSF could enhance xylanase production. e.g. *A. niger* KK2 produced cellulases and hemicellulases under SSF using different ratios of rice straw and wheat bran (Kang et al., 2004). Also, when a mixture of corn cob and wheat bran was used, xylanase production from *A. niger* and *A. ochraceus* increased by 18% (Betini et al., 2009). In the same point, Yang et al (2001) used a mixture of corn straw and wheat bran for xylanase production. Also, Pang et al (2006) found that the use of sugar cane baggase and palm kernel cake mixture for xylanase production under SSF by *Trichoderma sp*. FETLc3-2 gave good yield of enzyme production.

The influence of co-cultivation of the three isolates on xylanase production

The positive effect of co-cultivations of fungi on the production of hydrolytic enzymes has been published previously (Gupte and Madamwar, 1997; Verma and Madamwar, 2002; Zhang et al., 2006). Therefore, several plate growth experiments were performed as described in materials and methods to describe the effect of mixed cultures on xylanase production. Results showed that, the three fungi had different radial expansion rates on solid culture medium. *A. flavus* and *E.purpurascens* were overall the fastest growing fungi with *A. flavus* being the faster than of the two, while *C. sphaerospermum* in particular grew slowly. *A. flavus* did grow around and through the other fungi, without a significant effect on the growth of the other fungus. The edges of the colonies of A. *flavus, C. sphaerospermum* and *E.purpurascens* would grow through each other in the zone where the colonies would meet when they were inoculated at a distance away from each other. Therefore, further studies were carried out with these three isolates to determine the influence of fungal co-cultivation on extracellular enzyme production by growing the selected fungal species alone or as mixed culture.

Many of the co-cultivations resulted in increased activity for several enzymes, but often not for all enzymes. This suggests that the co-cultivation does not trigger a general increase in protein secretion but rather induction of specific enzymes, These effects are likely also dependent on the carbon source used (Hu et al., 2011). This is consistent with the obtained results in (fig. 3) where, the co-culture of ,*A. flavus* and *C. sphaerospermum* on WB and SD mixture with 1:2 ratio enhanced the xylanase production by 45.22% and 52.45% in comparison to the use of *A. flavus* and *C. sphaerospermum* each alone, respectively. *A. flavus* and *E. purpurascens* on WB enhanced the xylanase production by 42.11% and 134.29% in comparison to the use of *A. flavus* and *E. purpurascens* each alone, respectively. *C. sphaerospermum* and *E. purpurascens* on WB and SD mixture with 1:2 ratio enhanced the xylanase production by 48.67% and 132.25% in comparison to the use of *C. sphaerospermum* and *E. purpurascens* each alone, respectively. Also Dhillon et al (2011) reported that, mixed cultures of *Aspergillus niger* and *Trichoderma reseei* SSF using rice straw supplemented with wheat bran in the ratio 3:2 resulted in higher FP cellulase, β-glucosidase, endoglucanase (CMCase) and xylanase activities, compared to the activities obtained using mono-cultures. Results illustrated graphically in Fig. (4) showed that the co-culture of *A. flavus, C. sphaerospermum and E. purpurascens* on WB:SD mixture with 1.5:1.5 ratio enhanced xylanase production (61.34±1.11 U/g) by 55.41%, 63.31% and 156.22% if compared to the use of each one alone, respectively.

Photo.1 Screening for xylanase production, A: negative result for xylanase activity (no clearing zone, control), B: (*A. flavus*), C: (*C. sphaerospermum*), and D: (*E. purpurascens*) positive results for xylanase activity (clearing zone around the enzyme extract hole).

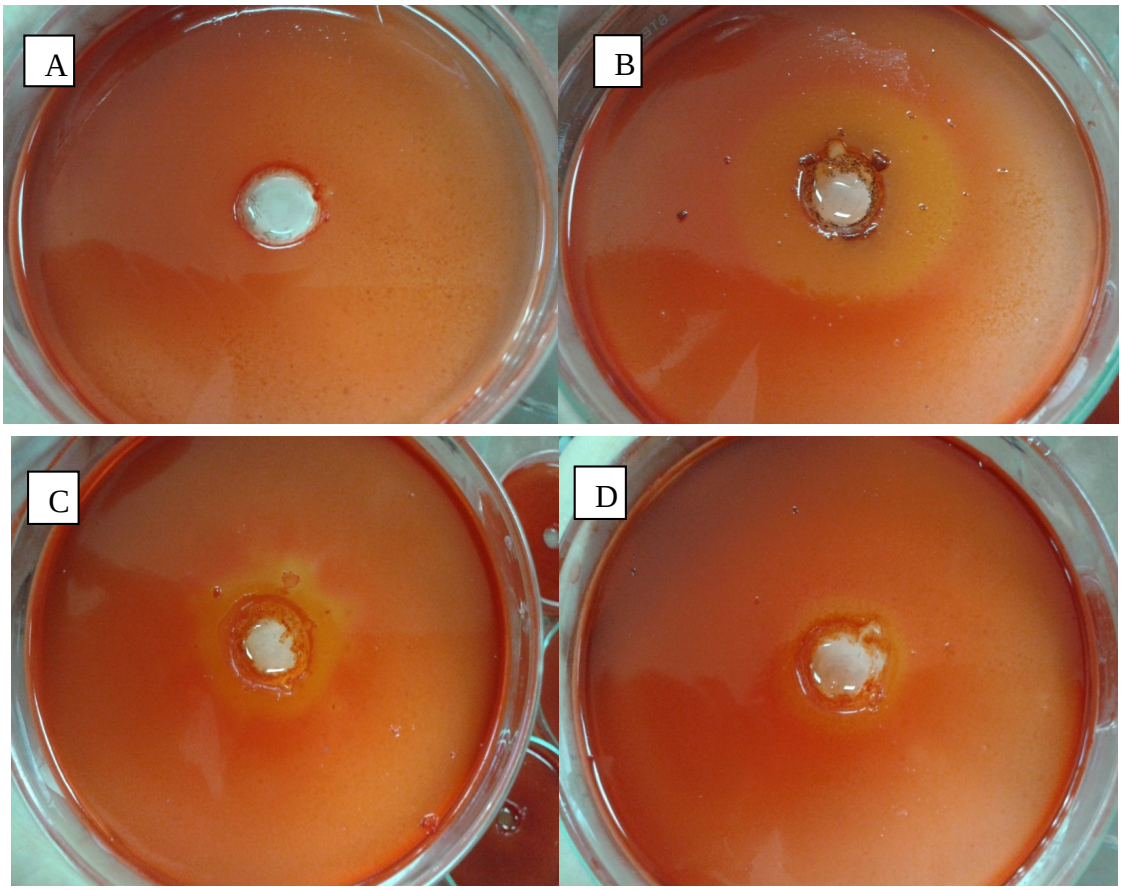


Fig.1 Xylanase production using different agroindustrial residues by *Aspergillus flavus*, *Cladosporium sphaerospermum* and *Epicoccum purpurascens.* Error bars correspond to standard deviation (three replicates)

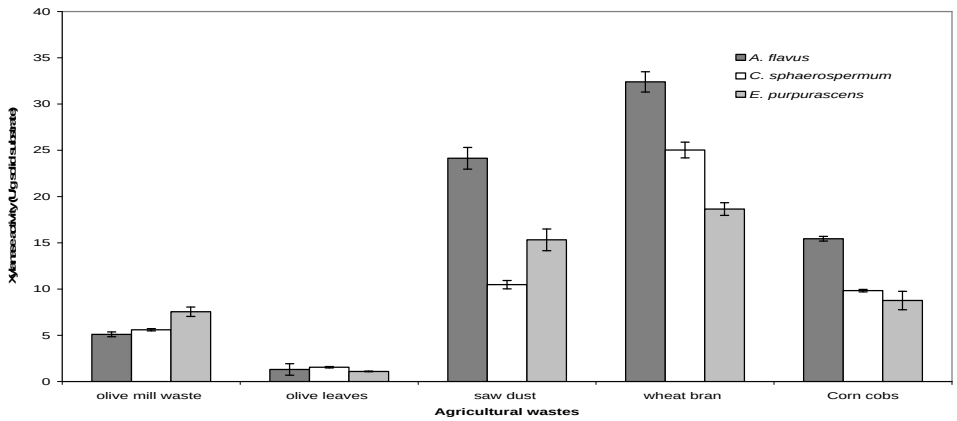


Fig.2 Effect of different proportions of wheat bran/Saw dust on production of xylanase
WB, wheat bran; SD, Saw dust

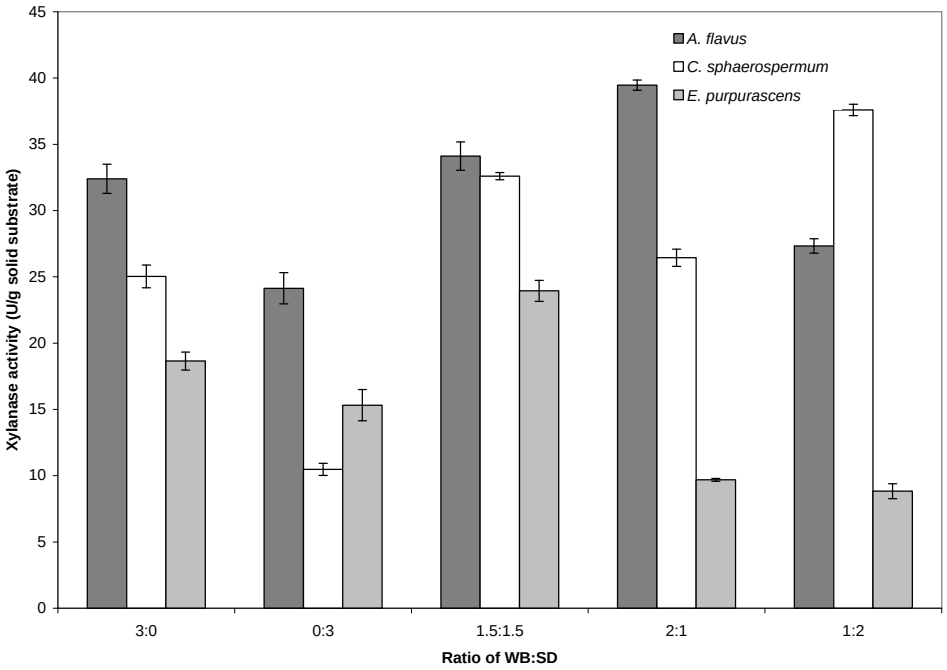


Fig.3 The influence of the co-culture of each two isolates on xylanase production

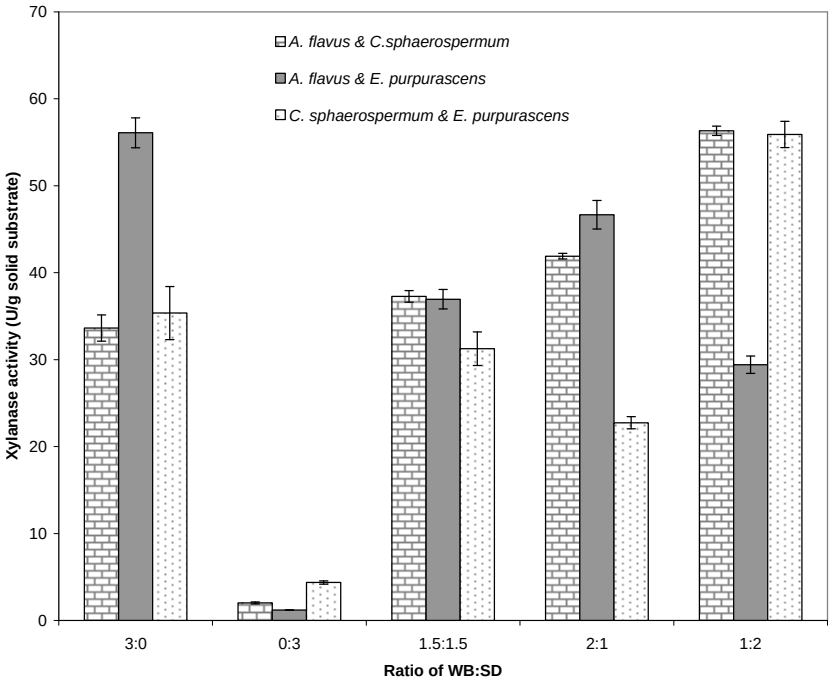


Fig.4 The influence of the co-culture of the three isolates on xylanase production

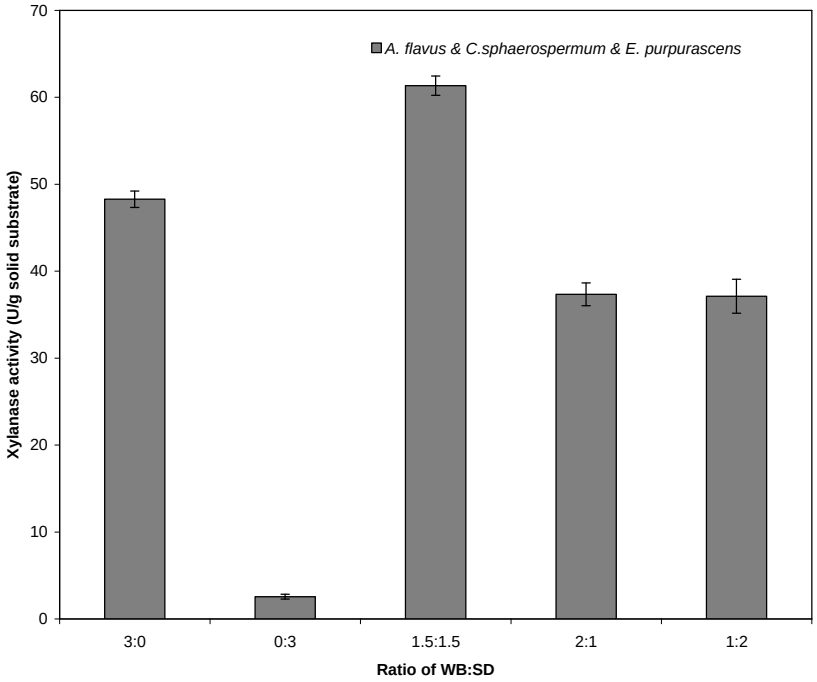
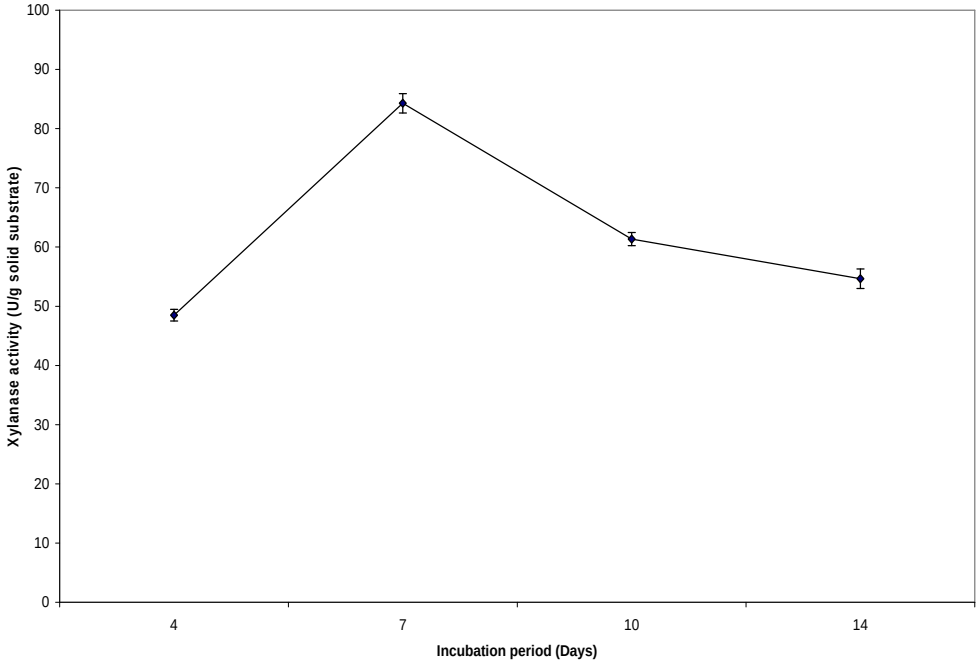


Fig.5 The influence of incubation time on xylanase production by the co-culture of *A. flavus, C. sphaerospermum and E. purpurascens*



Therefore, one of the attempts that made to increase xylanase production was the co-cultivation of *A. flavus, C. sphaerospermum* and *E. purpurascens* because the colonization of the substrate may be accomplished better in symbiotic association i.e. each species having its own niche for growth and substrate degradation.

In this connection, Kaushal et al (2012) enhanced xylanase production by the co-culture of *A. niger* and *Fusarium oxysporum* utilizing forest waste. Also, Rajesh & Rajesh (2012) obtained the enzyme xylanase from mixed culture of *Bacillus polymyxa* and *Cellulomonas uda*.

Time course of xylanase enzyme production by the co-culture of *A. flavus, C. sphaerospermum and E. purpurascens*

Environmental factors such as the incubation time considered one of the most important factors that affect the enzyme production by fungi (Sorgatto et al., 2012). Generally, the optimum fermentation period for maximum xylanase production during SSF was depending upon the nature of substrate, organism, additive nutrients and many other fermentable conditions (Dekker, 1983; Mishra et al., 1985). The results (fig. 5) showed progressive increase of xylanase production in the period 4-7 days of cultivation followed by a reduction. Probably, the reduction in xylanase yield was due to the depletion of available nutrients to micro-organisms, or due to the proteolysis (Sepahy et al., 2011). Also, Kavya and Padmavathi (2009) reported the highest xylanase production by *A. niger* under SSF on wheat bran after 6 days of incubation period. This coincides with that found by, Sater & Said (2001) with *Trichoderma harzianum* which gave the highest xylanase production after 8 days, and Seyis and Aksoz (2005) with *T. harzianum* 1073 D 3 after 7 days. Also, Simões et al (2009) and Rajesh and Rajesh (2012) reported the highest xylanase production after 6 days of incubation by *T. viride* and by mixed culture of *Bacillus polymyxa* and *Cellulomonas uda*, respectively.

In this study we investigated the feasibility of xylanase production by using low cost agricultural wastes for cheap and feasible enzyme production through simple and cheaper solid-state fermentation technology. In the present study, higher xylanase activity was obtained when wheat bran supplemented saw dust in the ratio 1.5:1.5, than the activities obtained using each one singly. And mixed culture combinations have the ability to utilize the substrates as energy sources better than the mono-cultures. In conclusion, mixed culture solid state fermentation of *A. flavus, C. sphaerospermum* and *E. purpurascens* using low-cost agro-wastes in the production of xylanase enzyme will ultimately bring down their production cost and at the same time reduce environmental pollution due to the wastes.

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