Screening of Microorganisms Isolated from some Enviro-Agro-Industrial Wastes in Saudi Arabia for Amylase Production

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Abstract: Wastes from food and drinks industries are becoming an increasing problem. The present study was focusing on the possibility of using different fermented enviro-agro-industrial wastes as very cheap and available substrates for obtaining microbial α-amylases that are of great industrial importance. Seventy three fungi and bacteria were isolated from twenty different wastes, e.g. food-industrial wastes, daily home wastes, expired foodstuff wastes, and some agricultural wastes from Al-Madinah Al-Munawwarah, KSA. Using these wastes as the sole carbon source, 38 fungi and 11 bacteria were isolated at 30 °C; 23 bacteria and one fungus were isolated at 55°C & 45°C, respectively. All isolates were then screened for α-amylase production and isolate *Aspergillus flavus*, F_2Mbb was selected as the most potent. Some environmental and nutritional parameters affecting the biosynthesis of α-amylases from *Aspergillus flavus*, F_2Mbb using bran as a sole carbon source were studied. It exhibited maximum amylase production at the following optimal conditions: incubation temperature, 37°C; pH, 6.0 using phosphate buffer; shaking condition at 200 rpm; incubation period, 6 days; inoculum size of 13.0×10^8 spore/ml on Modified Czapek-Dox wheat bran agar medium (MCDWB); a substrate concentration of 20% bran; chemical treatment of bran with 6 N phosphoric acid; carbon source, corn gluten; nitrogen source, $NaNO_3$; amino acid, methionine; and a mixture of different salts (100 ppm $MgSO_4$; 200 ppm KCl; 50 ppm K_2HPO_4 and 50 ppm $NiSO_4$). [Journal of American Science 2010;6(10):926-939]. (ISSN: 1545-1003).

Key words: Enviro-agro-industrial wastes, α-amylase production, *Aspergillus flavus*, *KSA*, environmental parameters, nutritional parameters.

1. Introduction:

Biomass generate large amounts of organic wastes: agricultural wastes, such as wheat straw, corn cobs, oat hulls, and sugarcane bagasse; residues from logging and timber milling; spoiled products and food-processing wastes; and urban solid waste such as paper, cardboard, kitchen and garden refuse (Glazer and Nikaido, 2007) and it is of vital importance that waste is managed in such a way that it does not cause any harm to either human health or to the environment (Rizvi, 2004).

Biodegradation is a breakdown of organic contaminants that occurs due to production of extracellular enzymes by microorganisms. These contaminants can be considered as the microbial food source or substrate (Maier *et al.*, 2000).

Enzymes have attracted attention of researchers all over the world because of wide range of physiological, analytical and industrial application; especially from microorganisms because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation (Chakrabortya *et al.*, 2009).

Amylases are hydrolytic enzymes that are widespread in nature, being found in animals, microorganisms and plants (Octávio *et al.*, 2000). With the advent of new frontiers in biotechnology, the amylase family enzyme finds potential application in a number of industrial processes such as bread making, brewing, starch processing, pharmacy, textile and paper industries (Pandey *et al.*, 2000, Alva *et al.*, 2007; Kammoun *et al.*, 2008). Amylases constitute a class of industrial enzymes representing approximately 25-33% of the world enzyme market (Nguyen *et al.*, 2002;Van der Maarel *et al.*, 2002) have almost completely replaced chemical hydrolysis of starch in starch processing industry (Pandey et al., 2000).

Each application of α-amylase requires unique properties with respect to specificity, stability, temperature and pH dependence (McTigue et al., 1995). Screening of microorganisms with higher α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications (Gupta *et al.*, 2003; Wanderley *et al.*, 2004). For these reasons, certain agricultural,

industrial and environmental wastes were investigated for their ability to induce α-amylase production.

The optimization of fermentation conditions, particularly physical and chemical parameters are of primary importance in the development of any fermentation process owing to their impact on the economy and practicability of the process (Wenster-Botz, 2000).

The purpose of this work was basically to examine the possible utilization of different enviro-agro-industrial wastes in KSA for α-amylase production. This purpose was achieved through different steps; isolation of α-amylase producing microorganisms from the different wastes; selection and identification of the most potent isolate producing the enzyme while utilizing the solid; and optimization of culture conditions affecting α-amylase biosynthesis by the preselected isolate.

2. Materials and Methods

Collection of samples

Twenty samples from food-industrial wastes (e.g, baggase and juice wastes), daily home wastes (white bread, brown bread, cooked chicken, cooked dough, cooked rice, macaroni and tea bags), expired foodstuff wastes (soya bean, white pepper, yellow lentils, brown lentils, seasam, white bean), and some agricultural wastes (palm leaves, banana) were collected from different districts of Al-Madinah Al-Munawwarah, KSA. Different samples from lab air were also collected.

All waste samples were dried, grinded and stored in sterile containers. One gram of each grinded waste was wet with sterile distilled water in small sterile tubes. A group of tubes were left for about 7 days in the incubator at 30 ºC for isolation of mesophilic fungi and bacteria. Another group was incubated at 45 ºC for five days for isolation of thermophilic fungi, and the third group at 55 ºC for three days for isolation of thermophilic bacteria. The fermented samples were considered as a source of microbial isolates.

Culture media

- *Czapek-Dox medium* (Czapek's, 1902 and Dox, 1910) was used in purification and maintenance of isolates. Furthermore, this medium can be used as a basal medium but without the addition of agar and sucrose. This medium contained (g/l): $NaNO_3$, 3; $K_2H\ PO_4$, 1; $MgSO_4\ .7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; agar, 15; sucrose, 20 at initial pH of 6.
- *Modified Czapek-Dox Waste Agar (MCDWA).* It was used as a primary isolation medium. Only 1% (w/v) of the previously prepared waste was used as the sole carbon source. It was used also as a production medium but without addition of agar.

Isolation and purification of amylolytic microbial cultures

Three groups of MCDWA medium and nutrient agar plates were prepared, one incubated at 30°C for isolation of mesophilic microorganisms, and the other groups incubated at 55°C and 45°C for isolation of thermophilic bacteria and fungi, respectively. The plates were routinely checked for any microbial growth for at least 7 days. All colonies of different forms and colors showing separate growth on Czapek-Dox medium or nutrient agar medium, were picked up; and technical purification steps were carried out.

Screening of microbial isolates for their amylolytic productivities

α-amylase assay

This was performed according to starch clearing zone (SCZ) technique of Elwan *et al.* (1986) standardized later by Ammar *et al.* (1998). The assay medium contained (g/l): starch, 10 and agar-agar, 15 and prepared according to the method of Gomori (1955). The activity of the α-amylase enzyme was estimated in terms of mean diameter of clearing zones (mm) using a special standard curve constructed for such a purpose and covering the range of 100 to 50000 µg/ml (Fig. 1).

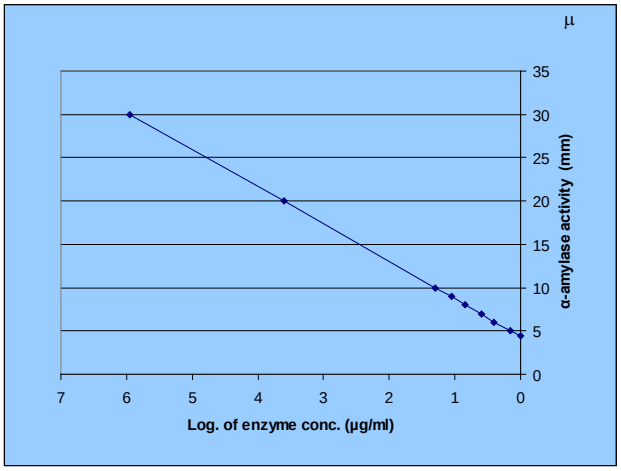

Figure 1. The standard curve of sigma α-amylase enzyme using starch clearing zone technique.

The production medium was prepared, inoculated by a specific isolate (each isolate was cultured on the same specified isolation medium), then incubated at the same isolation temperature for 6 days in case of fungi and two days for bacterial isolates. At the end of incubation, filtration was

carried out. The filtrate represented as a source of extracellular microbial α-amylase.

Characterization of the most potent isolate

The most potent isolate which shows the highest α-amylase productivity among all the mesophilic and thermophilic isolates, was selected for characterization. Its characterization was carried out in the Regional Center of Mycology and Biotechnology, Al-Azhar Univ., Cairo, Egypt. Isolate preliminarily identified, based on macroscopic and microscopic morphology on Czapek-Dox agar (CDA). The identification was made based on gross colony morphology and color and on microscopic features (magnification of 100× and 400×) in lactophenol cotton blue-stained wet mounts and according to international key.

Optimization of medium and culture conditions for α-amylase productivity by the selected isolate

The following factors were done to elucidate the best favorable conditions for the production of α-amylase by the most potent organism.

The strain was propagated on Czapek-Dox slants at 30 °C and monthly transferred. Stock cultures were maintained in spore suspension frozen in 25% glycerol (Djekrif-Dakhmouche *et al.,* 2006). Inocula in each case (triplicate sets) was prepared on 250 ml flask with 50 ml MCDW medium. Spores were suspended under agitation with a magnetic stirrer and counted by haemocytometer. Incubation was performed at 30 °C for 7 days. The deduced optimal condition resulted from each experiment was taken into consideration in the subsequent parameters. At the end of incubation period, the triplicate samples were harvested and the cells were separated by centrifugation. The supernatant was used for enzyme assay as determined before.

Many parameters were studied as: Different incubation temperatures in two types of media: MCDPF and MCDWB that contain crushed palm leaves and white beans, respectively; pH using 2 buffers (Phosphate buffer & Citrate phosphate Buffer) covering a pH range of 3.5-7.5; different incubation periods, different concentrations of wheat bran waste; Different inocula from spore suspension; different carbon sources; Different N-sources; and different salts.

3. Results and Discussion:

The possibility of depuration of wastes from food industry, by using them as substrates for various bioproductions of potential economic interest has been reported before (Murado *et al.,* 1993; Roukas, 1999; Guerra & Pastrana, 2003). The biological treatment of wastes was found to be an efficient way for reducing their initial chemical oxygen demand (COD) by more than 90% (Murado *et al.,* 1993).

Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides (Alva *et al.,* 2007). Microbial amylases meet industrial demands; a large number of them are available commercially (Kathiresan and Manivannan, 2006). Enzymes from fungal and bacterial sources have dominated applications in industrial sectors. Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* and *Penicillium* species (Kathiresan and Manivannan, 2006; Alva *et al.,* 2007).

In our study, Seventy-three microbial isolates were isolated from the twenty different waste samples (Tables 1,2). These isolates were screened for their ability to produce amylase. As a result of this study, we were determined many isolates having the ability to produce amylase at different levels. In the present investigation, 38 fungal isolates were isolated from different wastes and from air of the lab under mesophilic condition (30°C). All mesophilic fungal isolates were positive for α-amylase production (0.1-158.5 mg/ml) and the most potent isolates were F_2Mbb, F_5Mcd, F_6Mcr, F_{20}Mse, F_{32}Mla, F_{33}Mla, F_{34}Mla and F_{36}Mla (Table 1). Out of the 11 mesophilic bacterial isolates, 6 were α-amylase producers and the most potent isolates were B_4M bl and B_9M wbe.

Among the 24 thermophilic isolates under study, only three bacterial isolates (B_{13}Tcd, B_{19}Twbe, B_{23}Tyl) and the fungal isolate (FT pl) were exhibited the highest α-amylase production (Table 2).

In the present investigation, reevaluation of the most potent 14 mesophilic and thermophilic isolates (B_4M bl, B_9M wbe, F_2M bb, F_5M cd, F_6M cr, F_{20}M se, F32M la, F_{33}M la, F_{34}M la, F_{36}M la, B_{13}T cd, B_{19}T wbe, B_{23}T yl and FT pl) regarding α-amylase production was carried out on different food stuff wastes (Brown bread BB, Cooked rice CR, Soya bean SB, Palm leaves PL, White bean WBe and Wheat bran WB). The mesophilic isolate F_2Mbb had the ability to grow on different enviro-agro-industrial wastes with high α-amylase activity and it was selected as the most potent α-amylase producer. Moreover, wheat WB bran was the best waste that stimulated the highest enzyme productivity followed by PL and was selected for its availability and simplicity to manipulate it.

Fungal isolate F_2Mbb was found belong to *Aspergillus flavus*, so it was given the name and the code no (*Aspergillus flavus*, F_2Mbb). Figure 2 shows a microscopic picture by phase contrast microscope of it. This strain was maintained for using at the advanced experiments such as amylase production.

Table 1. Isolation of mesophilic microbes from different wastes and their α-amylase activity.

Bacterial B)/Fungal (F) code no	Isolation place (waste)	Isolation medium	α-amylase production (mg/ml)	Bacterial B)/Fungal (F) code no	Isolation place (waste)	Isolation medium	α-amylase production (mg/ml)
B_1M bw	Banana Waste (BW)	MCD BW	2.2	B_4M bl	Brown Lentils (BL)	MCD BL	89
B_2M bb	Brown Bread (BB)	MCD BB	21.2	B_5M bl	Brown Lentils (BL)	MCD BL	5.6
B_3M bb	Brown Bread (BB)	MCD BB	5.6	B_6M bl	Brown Lentils(BL)	MCD BL	0
B_7M cc	Cooked Chicken (CC)	MCD CC	0	B_9M wbe	White Bean (WBe)	MCD WBe	33.6
B_8M jw	Juice Waste (JW)	MCD JW	0	B_{10}M wp	White Pepper (WP)	MCD WP	0
B_{11}M yl	Yellow Lentils (YL)	MCD YL	0	F_{20}M se	Seasam (Se)	MCD Se	79.4
F_1M b	Baggase (B)	MCD B	0.3	F_{21}M se	Seasam (Se)	MCD Se	3.1
F_2M bb	Brown Bread (BB)	MCD BB	119.1	F_{22}M sb	Soya Bean (SB)	MCD SB	44.7
F_3M bb	Brown Bread (BB)	MCD BB	5.01	F_{23}M t	Tea Bags (T)	MCD T	22.4
F_4M cc	Cooked Chicken (CC)	MCD CC	1.1	F_{24}M t	Tea Bags (T)	MCD T	19.1
F_5M cd	Cooked Dough (CD)	MCD CD	63.1	F_{25}M wbr	White Bread (WBr)	MCD WBr	28.2
F_6M cr	Cooked Rice (CR)	MCD CR	79.4	F_{26}M wbr	White Bread (WBr)	MCD WBr	19.1
F_7M jw	Juice Waste (JW)	MCD JW	1.1	F_{27}M wp	White Pepper (WP)	MCD WP	0.8
F_8M jw	Juice Waste (JW)	MCD JW	19.1	F_{28}M wp	White Pepper (WP)	MCD WP	1.1
F_9M ls	Leaf Surface (LS)	MCD LS	0.1	F_{29}M yl	Yellow Lentils (YL)	MCD YL	3.1
F_{10}M ls	Leaf Surface (LS)	MCD LS	0.1	F_{30}M la	Lab Air (LA)	CDM	3.1
F_{11}M mw	Macaroni Waste (MW)	MCD MW	3.1	F_{31}M la	Lab Air (LA)	CDM	28.2
F_{12}M m	Mallow (M)	MCD M	8.9	F_{32}M la	Lab Air (LA)	CDM	100
F_{13}M m	Mallow (M)	MCD M	0.6	F_{33}M la	Lab Air (LA)	CDM	100
F_{14}M mnw	Mango Waste (MnW)	MCD MnW	0.6	F_{34}M la	Lab Air (LA)	CDM	158.5
F_{15}M mnw	Mango Waste (MnW)	MCD MnW	0.3	F_{35}M la	Lab Air (LA)	CDM	3.2
F_{16}M pl	Palm Leaves (PL)	MCD PL	0.5	F_{36}M la	Lab Air (LA)	CDM	63.1
F_{17}M pl	Palm Leaves (PL)	MCD PL	25.1	F_{37}M la	Lab Air (LA)	CDM	3.1
F_{18}M se	Seasam (Se)	MCD Se	2.5	F_{38}M la	Lab Air (LA)	CDM	19.1
F_{19}M se	Seasam (Se)	MCD Se	0.3				

Table 2. Isolation of thermophilic bacteria and fungi from different wastes and their α-amylase activity.

Bacterial B)/Fungal (F) code no	Isolation place	Isolation medium	α-amylase production (mg/ml)
B_1T b	Baggase (B)	MCDY B	1.1
B_2T b	Baggase (B)	MCDY B	19.1
B_3T b	Baggase (B)	MCDY B	0
B_4T bw	Banana Waste (BW)	MCDY BW	0.4
B_5T bw	Banana Waste (BW)	MCDY BW	0.5
B_6T bb	Brown Bread (BB)	MCDY BB	19.1
B_7T bb	Brown Bread (BB)	MCDY BB	7.1
B_8T bl	Brown Lentils (BL)	MCDY BL	0.5
B_9T bl	Brown Lentils (BL)	MCDY BL	0.5
B_{10}T cc	Cooked Chicken (CC)	MCDY CC	3.1
B_{11}T cc	Cooked Chicken (CC)	MCDY CC	15.9
B_{12}T cc	Cooked Chicken (CC)	MCDY CC	19.1
B_{13}T cd	Cooked Dough (CD)	MCDY CD	**177.8**
B_{14}T cr	Cooked Rice (CR)	MCDY CR	0
B_{15}T cr	Cooked Rice (CR)	MCDY CR	0
B_{16}T cr	Cooked Rice (CR)	MCDY CR	0.5
B_{17}T cr	Cooked Rice (CR)	MCDY CR	0
B_{18}T wbe	White Bean (WBe)	MCDY WBe	0
B_{19}T wbe	White Bean (WBe)	MCDY WBe	**89.1**
B_{20}T wbe	White Bean (WBe)	MCDY WBe	0.4
B_{21}T wbr	White Bread (WBr)	MCDY WBr	1.8
B_{22}T yl	Yellow Lentils (WL)	MCDY YL	0.8
B_{23}T yl	Yellow Lentils (WL)	MCDY YL	**141.3**
FT pl	Palm leaves (PL)	MCDY PL	**33.6**

B: bacteria, T: thermophilic, F: fugus, MCD: Modified Czapek-Dox medium. Y: Yeast extract

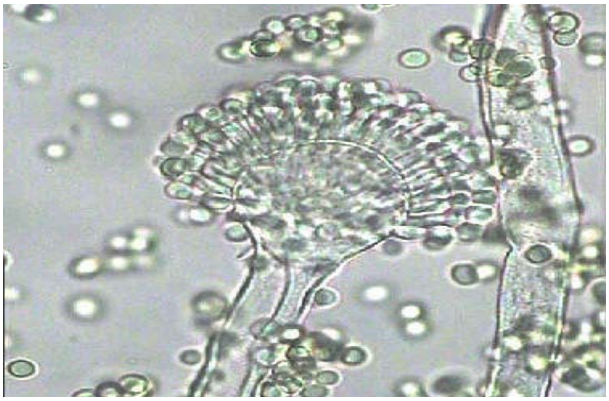

Figure 2. A microscopic picture by phase contrast microscope of the most potent fungal isolate *Aspergillus flavus*, F_2Mbb.

Many microbial species have already been identified as good amylase producers. Studies of amylases from bacteria and fungi are well documented (Dey *et al.*, 1991; Babu and Satyanarayana, 1995; Murado *et al.*, 1997; Sidhu *et al.*, 1997; Coronado *et al.*, 2000; Yuguo *et al.*, 2000; Jin *et al.*, 2001; Stamford *et al.*, 2001). The *Aspergillus* species produce a large variety of extracellular enzymes of which amylases and proteases are of significant industrial importance (Pandey *et al.*, 2000).

Also, Abou-Zeid (1997) screened filamentous fungi isolated from cereals for their ability to produce alpha-amylase (1,4-alpha-glucan glucanohydrolase, EC 3.2.1.1) and the selected strain was identified as *Aspergillus flavus* showed high enzymatic activity.

The environmental and nutritional parameters controlling α-amylase production by the most potent fungal isolate F_2Mbb on Czapek-Dox palm leaves agar medium MCDPL & Czapek-Dox wheat bran agar medium MCDWB were investigated. It is clear that 37 °C was the optimum temperature for the highest α-amylase production by *Aspergillus flavus*, F_2Mbb for both types of substrates as shown in Table 3.

The present findings are in accordance with other authors who found that the optimum temperature for amylase production was in a range between 25 and 37 °C for the mesophilic fungi (Ueno *et al.*, 1987; Gupta *et al.*, 2003; Kathiresan and Manivannan, 2006). Moreover, Sani *et al.* (1992) indicated that, *Aspergillus flavus* and *Aspergillus niger* produce extracellular amylase into the culture medium at maximum activity at the growth temperature of 29.0±1°C. Recently, Shafique *et al.* (2010) determined that 30 °C was the optimum temperature for maximum alpha-amylase enzyme activity for *Aspergillus niger* FCBP-198.

Concerning the effect of different incubation time by the most potent fungal isolate, the fourth and sixth day of incubation exhibited the highest α-amylase production with MCDPL & MCDWB respectively (Table 4). In accordance to this result, the highest enzyme production was obtained after 4 days by *A. niger* (Al-Azhary *et al.* $1984_{a,b}$ and Pandey 1991); and by *Aspergillus flavus* var. *columnaris* (Ammar and El-Safey, 2003).

The medium MCDWB was chosen to complete the present work because it gave better results than MCDPL in the previous two experiments.

In the present investigation, shaking condition proved to play a role in the α-amylase biosynthesis by the most potent fungal isolate *Aspergillus flavus*, F_2Mbb when allowed to grow on MCDWB (Table 5). In view of the findings of other investigators, alpha-amylases could be produced under the submerged culture condition (Bose and Das, 1996; Egas *et al.*, 1998; Aguilar *et al.*, 2000). Also, Gupta *et al.* (2003) reported that, agitation intensity influences the mixing and oxygen transfer rates in much fungal fermentation and thus controls mycelial morphology and ∝-amylase formation. In addition, Olama and Sabry (1989) reported that, the maximal amylase productivity of the two mold fungi *A. flavus* and *P. purpurescence* when grown on starch-containing medium in shaked flasks.

However, Kammoun *et al.* (2008) indicated that, the speed of agitation shows limited positive effect on the enzyme production. An increase of the agitation, on such limits, causes an enzyme production enhancement since the aeration is an important factor for the growth of aerobic strains. According to Amanullah *et al.* (2000); Djekrif-Dakhmouche *et al.* (2006), α-amylase production is independent of the speed of agitation. In the investigation of Gonzalez *et al.* (1992), a rotary speed of agitation at 200 rpm has caused an increase in the α-amylase production of *A. niger*.

According to Soni *et al.* (1992), the synthesis of the extra-cellular α-amylase is affected by pH, just like its secretion and the stability of the amylolytic system (Fogarty, 1983).

By studying the effect of buffers of different pH (Table 6), a pH 6 of phosphate buffer induced the highest productivity of α-amylase production by F_2Mbb. This is in accordance with De-Mot and Verachtert (1986) who reported that the majority members of Aspergilli producing amylases required acidic media (3.0-6.5). Moreover, optimal amylase production was obtained at pH 5.0 by *Aspergillus ochraceus* (Nahas and Waldemarin, 2002); pH 4 by *Aspergillus flavus* var. *columnaris*, s-9KP (Ammar and El-Safey, 2003); pH 4.5 by *Aspergillus niger*

FCBP-198 (Shafique *et al.*, 2010); the variation in the pH from 5 to 6 has a positive effect on the production of both α-amylase by the *Aspergillus niger* ATCC 16404 fungus (Djekrif-Dakhmouche *et al.*, 2006).

Concerning the different inoculum size (Table 7), an inocula of 13 x 10^8 spore/ml induced the highest α-amylase production by the most potent fungal isolate F_2Mbb on MCDWB. Francis *et al.* (2003) and Jiff *et al.* (1998) reported that inoculum size was a parameter which affects greatly the production of α-amylase in *A. oryzae*. Similarly, Mulimani *et al.* (2000) reported that inoculum volume per gram of substrate was among the most effective parameters in stimulating amylase production by *Gibberella fujikuroi.*

Similarly, a concentration of $3x10^8$ spores/ml exerted the highest amylolytic activity of *Aspergillus flavus*, F7 (Sidkey *et al.*, 1996); 0.0637 x 10^6 /ml^{-1} of *Aspergillus flavus* var. columnaris, s-9KP (Ammar and El-Safey (2003); 5 $x10^6$ spores per flask were used for enzyme production by *Aspergillus niger* ATCC 16404 (Djekrif-Dakhmouche *et al*., 2006)

Concerning the relation between different chemical treatments of the MCDWB (viz: HCl, H_2SO_4, H_3PO_4 and NH_3) and α-amylase production by the most potent fungal isolate, phosphoric acid was the best treatment among the other treatments used in this study (Table 8). Doelle *et al*. (1992) reported the ability to pre-treat (chemically or mechanically) some of the substrates before using in solid state fermentation (SSF) processes, making them more easily accessible for microbial growth. Also, Alani and Smith (1988) and Doran *et al. (*1994) showed that, the pre-treatment of agricultural waste has often been found useful to improve its digestibility and easy access for microbial attack (by removing core and noncore lignin fractions).

Doelle *et al*. (1992) indicated that, the selection of a substrate for enzyme production depends upon several factors, mainly related with cost and availability of the substrate, and thus may involve screening of several agro-industrial residues.

Many literatures were obtained in this respect, Pandey (1990) reported that, wheat bran however holds the key, and has most commonly been used, in various processes.

Growth on wheat bran gave the highest amylase activity in SSF by *Bacillus coagulans* B49 (Babu and Satyanarayana, 1995); *Thermomyces lanuginosus* (Kunamneni *et al.*, 2005); *Aspergillus oryzae* using 14 agro-industrial wastes (Sivaramakrishnan *et al.*, 2007).

A concentration of 20% of wheat bran exhibited the highest α-amylase productivity. Above or below that concentration a sharp decrease was noticed (Table 9). However, Djekrif-Dakhmouche *et al*. (2006) indicated that, the production medium containing orange waste powder (2% w/v) favoring enzyme production by *Aspergillus niger* ATCC 16404. Moustafa (2002) proved that, a concentration of 20% (g/l) water hyacinth ground preparation as the sole carbon source stimulated the highest amylolytic activity for both fungal and bacterial isolates *Thermomyces lanuginosus*, F_4 and *Streptobacillus moniliformis*, B_7, respectively.

Concerning the effect of different carbon sources on α-amylase production by the most potent fungal isolate F_2Mbb (Table 10), starch soluble followed by the corn gluten were the best followed by the disaccharides lactose and galactose. The results indicated that, the produced α-amylase enzyme is an inducible enzyme. Corn gluten was preferred in this study as it is considered a carbon as well as a nitrogen source. This is in accordance to Djekrif-Dakhmouche *et al*. (2006) who recorded that the addition of starch during fermentation increases the α-amylase. The α-amylase synthesis necessitates therefore, the presence of starch, an enzyme substrate, because of its inductive effect (Tani *et al.*, 2000; Ray, 2001), its remarkable efficiency in the production of enzyme, being an inexhaustible source of carbon compared to other carbon sources (Le Mense *et al.*, 1947; McTigue *et al.*, 1994), and because of its role in stabilizing the enzyme (Santamaria *et al.*, 1999; Aguilar *et al.*, 2000).

Similarly, Olama and Sabry (1989) reported that, the two mold fungi *A. flavus* and *P. purpurescence* produce extracellular amylase when grown on starch-containing medium as a sole carbon source.

However, Ammar and El-Safey (2003) reported that, the carbon source for α-amylase production by *Aspergillus flavus* var. *columnaris*, s-9KP allowed to grow on natural substrates, was ribose for maize meal and lactose for rice husk under SSF conditions.

Regarding effect of different nitrogen sources on α-amylase production by the most potent fungal isolate F_2Mbb (Table 11), sodium nitrate followed by yeast extract exhibited the highest biosynthesis. In view of the findings of other workers of the nitrogen sources stimulated the highest yield of amylase were casamino acids, ammonium nitrate and sodium nitrate by *A. ochraceus (*Nahas and Waldemarin, 2002); and sodium nitrate by *Aspergillus flavus* var. *columnaris*, s-9KP (El-Safey and Ammar, 2002) and *Aspergillus niger* FCBP-198 (Shafique *et al.*, 2010).On the other hand, some investigators reported that organic nitrogen sources are preferred for the production of α-amylase. A maximum α-amylase production was supported by yeast extract; peptone or beef extract (Krishnan and

Chandra, 1982; Emanuilova and Toda, 1984; Hayashida *et al.*, 1988; Hamilton *et al.*, 1999). Recently, Kammoun *et al*. (2008) indicated that, the organic nitrogen source, like casein acid hydrolysate and soybean meal hydrolysate, have traditionally a positive effect over inorganic ones since they are also carbon sources and contain trace of minerals and ions that could enhance the enzyme secretion.

In this work, the sulfur containing amino acid methionine exhibited the highest enzyme productivity among the different 24 amino acids (Table 12).

On the other hand, Sidkey *et al*. (1996) found that the acidic amino acid groups (glutamic acid and aspartic acid) were the best sources for inducing the highest amylase productivity by *Aspergillus flavus*, S-7. Moustafa (2002) also observed the maximal α-amylase productivity with the acidic amino acids groups. These amino acids are represented by aspartic and glutamic acids in case of *Thermomyces lanuginosus*, F_4 while DL-alanine exhibited the highest α-amylase(s) productivity in case of *Streptobacillus moniliformis* B_7.

In the present investigation, to be sure of the proceeded results, different materials (viz; corn gluten, methionine and $NaNO_3$) which exhibited the highest α-amylase production by the most potent fungal isolate F_2Mbb were studied. The mentioned materials above were added separately or in combinations. A combination of methionine + corn gluten 0.5g + $NaNO_3$ was found to stimulate the highest α-amylase productivity when allowed to grow on wheat bran as the sole carbon source (Table 13).

Salts have a positive effect on the production of α-amylase (Table 14). These results support other investigations of the same enzyme belonging to other microbial species (whether fungal or bacterial) (Ilori *et al.*, 1997; Narang &Satyanarayana, 2001; Djekrif-Dakhmouche *et al*. 2006). By studying the effect of different salts, a concentration of 200 ppm of KCl, 50 ppm of K_2HPO_4, 100-200 ppm of $MgSO_4$ and 50 ppm of $NiSO_4$ were induced the highest α-amylase productivity by the most potent fungal isolate F_2Mbb.

Certain microorganisms are sensitive to microelements in the medium, resulting either in suppression or stimulation of growth and enzyme yield (Mohamed, 1976). Furthermore, the salts that gave the highest productivity were added together in a mixture. The mixing of the different salts with corn gluten (C), $NaNO_3$ (N) in the presence of amino acid (methionine) gave the highest α-amylase productivity by most potent fungal isolate as shown in Table 15.

Phosphate has a main regulatory role in the synthesis of primary and secondary metabolites in microorganisms and likewise it affects the growth of the organism and production of α-amylase (Gupta *et al.*, 2003). In view of the other findings, Kammoun *et al.* (2008) reported that, KH_2PO_4, $(NH_4)_2SO_4$, $CoCl_2$, $MgSO_4$ were selected based on their positive influence on α-amylase formation by *Aspergillus oryzae* CBS. They also elucidated that, phosphate and magnesium seem to play important roles on the expression of alpha amylase.

Table 3. Effect of different incubation temperatures on α-amylase production by the most potent fungal isolate F_2Mbb on MCDPL & MCDWB.

Incubation temperature (°C)	α-amylase production (mg/ml)	
	MCD PL	MCD WB
10	0	0
15	0	0
20	6.6	68.88
25	9.85	105.11
30	90.65	42.40
37	**156.2**	**247.00**
40	27.5625	31.89
45	1.15	1.49
50	0	0

MCDPL=Czapek-Dox palm leaves agar medium, MCDWB= Czapek-Dox wheat bran agar medium.

Table 4. Effect of different incubation time on α-amylase production by the most potent fungal isolate F_2Mbb on MCDPL & MCDWB.

Incubation time (Days)	α-amylase production (mg/ml)	
	MCD PL	MCD WB
2	202.875	199.9125
4	314.225	393.9125
6	63.1	**721.8875**
8	100	586.45
10	63.1	362.45

Table 5. Effect of static and shaking incubation on α-amylase production by the most potent fungal isolate F_2Mbb on MCDWB.

Incubation condition	α-amylase production (mg/ml)
Static	4.2125
Shaking	**2925.425**

Table 6. Effect of different pH on α.amylase production by the most potent fungal isolate F_2Mbb on MCDWB.

pH	α-amylase production (mg/ml)
3.6	7682.35
4.2	**8371.075**
4.8	5530.3
5.4	5917.6
6	**9796.5625**
6.5	8672.5
7	5851.725
7.5	4021.1

Table 7. Effect of different inculum size on α-amylase production by the most potent fungal isolate F_2Mbb on MCDWB.

Inoculum Size (spores/ml) x 10^8	α-amylase production (mg/ml)
3.5	11443.5375
6.5	9145.675
13	**16270.95**
26	9145.675
39	9021.9625
52	7682.35

Table 8. Effect of chemical treatment of the waste on α-amylase production by the most potent fungal isolate F_2Mbb on MCDWB.

Chemical Treatment (6N)	α-amylase production (mg/ml)
HCl	5097.125
H_2SO_4	2330.575
H_3PO_4	**5668.075**
NH_3	3496.375

Table 9. Effect of different substrate concentrations on α-amylase production by the most potent fungal isolate F_2Mbb on MCDWB.

Substrate concentration (%)	α-amylase production (mg/ml)
0.25	474.55
0.5	655.7
1	1197.45
2	1685.075
4	2708.95
8	2173.5
12	2043.2
16	3114.1
20	**9918.7**
30	3924.4

Table 10. Effect of different carbon sources on α-amylase production by the most potent fungal isolate F_2Mbb on MCDWB.

Carbon source	α-amylase production (mg/ml)
Control	16913.9
Galactose	21046.25
Dextrose	18291.35
Fructose	10286
Sucrose	16742.15
Glucose	17516.75
Lactose	23801.15
Maltose	18291.35
Starch soluble	**30701.075**
Starch insoluble	9144.1
Corn gluten	**27019.425**

Table 11. Effect of different nitrogen sources on α-amylase production by the most potent fungal isolate F_2Mbb on MCDWB.

Nitrogen source	α-amylase production (mg/ml)
$NaNO_2$	9511.4
$NaNO_3$	**19668.8**
NH_4HPO_4	14222.8
$(NH_4)_2SO_4$	13448.2
NH_4Cl	10326
NH_4 citrate	5943.3
Peptone	6493
Urea	12609.8
Yeast extract	**14458.35**

Table 12. Effect of different amino acids on α-amylase production by the most potent fungal isolate F_2Mbb on MCDWB.

Amino acid	α-amylase production (mg/ml)	Amino acid	α-amylase production (mg/ml)
Alanine	1782.6	L-leucine	5084.65
Amino buteric acid	4933.85	L-lysin-HCl	2825
Arginine-HCl	2825	**Methionine**	**11835.2**
Asparagine	2853.5	Ornithine	1782.6
Aspartic acid	4137.8	Phenyl alanine	1392.5
Cycteine-HCl	1782.6	Proline	1587.55
DL- alanine	1392.5	Serine	1782.6
DL-nor leucine	11060.6	Threonine	2564.4
Glutamic acid	6350.6	Tryptophan	1002.4
Glycine	6493	Tyrosin	1782.6

Hystidine-HCl	1782.6	Valine	3380.4
Iso-leucine	1782.6	Control	1392.5
L-cysteine	9348.25		

Table 13. Effect of different combinations of (corn gluten+$NaNO_3$+methionine) on α-amylase production by the most potent fungal isolate F_2Mbb on MCDWB.

Carbon +Nitrogen +Amino acid	α-amylase production (mg/ml)
Control (no addition of C or N or amino acids.)	6992.75
Corn gluten 0.5 g	7267.6
Corn gluten 1 g	5483.55
Methionine	4841.4
$NaNO_3$	5758.4
Corn gluten 0.5 g + Methionine	11060.6
Corn gluten 0.5 g +$NaNO_3$	8776.8
Methionine + $NaNO_3$	16570.4
Methionine + Corn gluten 0 .5 g + $NaNO_3$	**17676.625**

Table 14. Effect of different salts on α-amylase production by the most potent fungal isolate F_2Mbb on MCDWB.

Minerals (ppm)	α-amylase production (mg/ml)	Minerals (ppm)	α-amylase production (mg/ml)	Minerals (ppm)	α-amylase production (mg/ml)
$AgNO_3$		**$CdSO_4$**		**$MgSO_4$**	
50	8739.65	50	2853.5	50	3388.95
100	12732.525	100	13041.6	100	**25178.6**
200	10486.55	200	12638.6	200	**25178.6**
$CaCl_2$		**$CuSO_4$**		**$MnSO_4$**	
50	2592.9	50	16225.65	50	10062.5
100	2853.5	100	5758.4	100	2555.05
200	9014.5	200	21046.25	200	9252.2
$CaHPO_4$		**$FeSO_4$**		**$NiSO_4$**	
50	6900.3	50	20874.5	50	**25178.6**
100	11116.6	100	5644.85	100	1069.1
200	11764.325	200	20874.5	200	16570.4
$Ca(OH)_2$		**KCl**		**Control**	4566.55
50	4545.1	50	10114.25		
100	20735.025	100	4872.4		
200	11899	200	**25178.6**		
$CaSO_4$		**K_2HPO_4**			
50	3979	50	**25178.6**		
100	13080.9	100	13316.45		
200	9184.1	200	3388.95		

Table 15. Effect of different combinations on α-amylase production by the most potent fungal isolate F_2Mbb on MCDWB.

Minerals concentration (ppm)	α-amylase production (mg/ml)
Control + C& N sources (without salts & methionine)	16977.7
Salts + C& N sources	39905.2
Salts + C& N sources + Methionine	**54773.9**

Where salts= (100 ppm $MgSO_4$; 200 ppm KCl; 50 ppm K_2HPO_4 and 50 ppm $NiSO_4$), C source: corn gluten, N source: $NaNO_3$

According to bibliographic studies, the effects of P^- and Cl^- ions on the stability of α-amylase have been well recognized (Chessa *et al.*, 1999; Pandey *et al.*, 2000).

Kundu *et al.* (1973) indicated that, Ca^{2+} was inhibitory to amylase production by *A. oryzae* EI 212 although, Ca^{2+} has been reported as essential for alpha amylase stability (Gupta *et al.*, 2003). In addition, McTigue *et al.* (1994) indicated that both calcium and manganese are necessary for the α-amylase biosynthesis. Also Djekrif-Dakhmouche *et al*. (2006) proved that the use of manganese, iron sulfates as well as magnesium chloride seems to increase the production of α-amylase,

In conclusion, the present data focusing on obtaining microbial isolates which have the ability to use different enviro-agro-industrial wastes as very cheap and available substrates for obtaining fungal amylases. An interesting scope for further research would be to use the optimized medium for the synthesis of *Aspergillus flavus*, F_2Mbb α-amylase, characterization and the investigation of enzyme kinetics for determination of industrially availability.

Acknowledgement

This work was supported by grant No. 428/145 from Deanship of scientific Research, Taibah University, KSA.

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