Radiation mutagenesis and purification of xylanase produced by soil fungi

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Abstract

The production, optimization, radiation mutagenesis and purification of extracellular xylanase enzyme produced by some fungal species isolated from three soil sites located in Jeddah governorate, Saudi Arabia were studied. Ten fungal species (*Alternaria alternata, Aspergillus candidus, Aspergillus ochrochus, Aspergillus flavus, Botrytis cinerea, Fusarium roseum, Penicillium chrsogenum, Penicillium italicum, Penicillium canescens* and *Rhizopus microsporus*) constituting 188 colonies were isolated. The highest significant value of extracellular xylanase enzyme (0.60 unit/ml) was shown in the culture filtrate of the *Aspergillus candidus*. Optimization of some nutritional and physical factors in order to intensify the production of *A. candidus* extracellular xylanase was carried out. The highest productivity of *A. candidus* extracellular xylanase (0.60 and 0.62 unit/ml) occurred on xylan and peptone as carbon and organic nitrogen sources, respectively. The optimum pH and temperature for extracellular xylanase activities were 9.0 and 55 °C, respectively. The effect of ultraviolet radiation on *A. candidus* xylanase indicated that the maximum stimulation of *A. candidus* xylanase (1.20 unit/ml) was attained on exposure to UV after 50 min. The radio-stimulated xylanase from the most xylanase producing organism (*A. candidus*) was purified to homogeneity by salting out with ammonium sulphate, dialysis and passage through chromatography resins (Sephadex G-100 column and Diethylaminoethyl sephadex column). The irradiated purified xylanase enzyme resulted in 1.301 fold of purification over the crude extract, exhibited a specific activity of 2.258 unit/mg with the recovery of 90.5 %. Test for the purity by Sodium dodecyl sulfate polyacrylamide gel electrophoresis technique (SDS-PAGE) resulted in a single protein band of the pure irradiated xylanase. Studying factors affecting the activity of the irradiated purified xylanase was carried out. The optimum reaction temperature and pH for maximum purified xylanase activities (1.82 and 2.10 unit/ml) were 60°C and 8, respectively. Thermostability of the irradiated purified *A. candidus* xylanase showed that it was stable (35% loss of activity) a 60°C after 60 min. exposure time.

Keywords: Xylanase, Aspergillus, Purification, Characterization.

INTRODUCTION

The last few decades expressed on active scientific and technological development of research on ultilization of waste materials as a renewable base in the last few decades. Lignocelluloses bioconversion as a renewable energy source. Xylan is the major constituent of hemicelluloses and has a high potential for degradation to useful products. These compounds were present in the cell wall and in the middle lamella of plant cells. Xylanase the main enzyme using for de-structure of the xylan backbone was xylanase. It hydrolyze randomly on the backbone of xylan to make shorter chain monosaccharide as xylose (Khucharoenphaisan and Sinma, 2010).

Chemical hydrolysis of xylan applied extensively by the industries, although the proces was faster, it is accompanied with the formation of toxic compounds and is hazardous to the human health and environment (Ninawe *et al*., 2008).

Microorganisms are a treasure of useful xylanase enzymes because they multiply at a high rate and

synthesize biologically active products, which humans can control. Enzymes now are used as industrial catalysts for many reasons: they exhibit high catalytic activity and a high degree of substrate specificity, can be produced in large amounts, are highly biodegradable, pose no threat to the environment and economically viable. Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial applications, like xylanase in fungi (Sarao *et al.,* 2010).

extensive research on fungi has been conducted to isolate new organisms with large secretion of ligninolytic enzymes as well as enzymes with potential industrial applications. Xylanases are one of the enzymes with lignin-degrading ability (Altaf *et al.,* 2010).

Xylanase acts on β-1,4 xylan and cleaves β-1,4 glycosidic linkage randomly. Xylanase enzyme has attracted considerable research interest because it increasing the body mass of the animal (Silversides and Bedford, 1999) and is used in food industry (Figueroa-Espinoza *et al.*, 2004) in paper manufacturing to degrade xylan and to increase the brightness of pulp and to clarify fruit juices was found by Garg *et al.* (2009).

Using xylanase avoids the use of chemical processes that are expensive and cause pollution. The chemical extraction of lignin is improved by xylanases treatment. Understanding physiological mechanism that regulates enzyme synthesis and purification could be useful for improving the technological process of enzymes production (Songulashvili *et al.*, 2007).

Effects of electromagnetic radiation on biological systems have spurred a great research of its biological interest and technological applications (Trombert *et al*., 2007).

Geweely *et al.* (2006) stimulated alkalo- thermophilic *Aspergillus terreus* xylanase by low intensity radiation. Characterization of the purified enzymes revealed that the enzyme recovered from the irradiated fungus was more thermostable and had a wider range of optimum pH values (4.0-12) and temperatures (60-70 ºC). The xylanase enzyme recovered from irradiated alkalo-thermophilic *A. terreus* is preferable than the non-irradiated. Santiago *et al.* (2006) stated that the mutant strains from *Aspergillus niger* were produced by UV radiation to increase their enzyme activity production.

The main aim of the present work is to search for new sources of fungal xylanase isolated from different soil samples located in Jeddah district, Saudi Arabia. The fungal isolates were evaluated under different conditions. That will be to select the most efficient fungal species producing xylanase. We will be consider cultural and physical factors that influencing the induction of extracellular xylanase enzyme. Radiation mutagenesis by ultraviolet will be carried out for stimulating the production of extracellular xylanase. Extraction and purification of the most efficient radio-stimulated fungal species producing xylanase to characterize and specify it for possible use in biotechnology.

MATERIAL AND METHOD

Collection of soil samples

Seven soil samples were collected from three different sites located in Jeddah district, Saudi Arabia. These sites are: Makkah road (Makkah high way, (10 Km east of Jeddah district and 40 Km south of Jeddah district), Al-Madina Al-Monawara road (Al-Madina high way, 30 Km north of Jeddah district and Obhur way, 50 Km north of Jeddah district) and three agriculture soil sites, 20 Km east of high way [*Ocimum basilicum* (Ocimum) agriculture soil, *Ziziphus spina-christi* (Ziziphus) agriculture soil and *Phonix canariensis* (Date Palm) agriculture soil]. The samples were taken at a depth of 20 cm from the soil surface according to the method described by Johnson *et al.* (1959).

The culture media

Xylan medium

Xylan 10.0, Peptone 5.0, Potassium dihydrogen phosphate 1.0, Magnesium sulfate 0.5, Agar-agar 20.0. After solubilization, streptomycin 30 μg/ml and rose bengal 1:30000 were added to prevent bacterial growth.

Determination of soil fungi

Soil samples were gathered according to the method described by Abou-Zeid and Abd El-Fattah, (2007). Five soil samples were taken into plastic bags. Samples are brought together into one composite sample, kept in cool place during transportation and storage. They were used to determination fungal count by dilution plate method. Soil diluted, sieved and the dry soil was placed in a graduated cylinder. Water was added till the total volume was 250 ml. The suspension was stirred and poured into 1000 ml Erlenmeyer flask and shaked for 30 min. Ten ml of this suspension were withdrawn (while in motion) with a sterile 10 ml pipette and transferred into immediately through successive 90 ml sterile water blank till the desired final dilution (10^{-4}) was reached. Suspensions were shaken by hand for seconds. Pipetting was carried out while the suspension was in motion. One ml of the desired dilution was transferred into each of several Petri-dishes (3 dishes), then 12-15 ml of an appropriate xylan medium cooled above the solidifiying temperature, added to each dish. The dishes were rotated by hand in a

swirling motion so the soil suspension was dispersed in the agar. After incubation at 25 ^{o}C from 5 to 7 days we counted the resulting colonies. The average number of colonies was multiplied by the dilution factor to obtain the number per gram of the original soil.

Purification of the isolated fungal species

The isolated fungal species were purified by streak plate method. After incubation for 7 days at 25 °C, a single colony was aseptically sub cultured on a slant of xylan medium.

Identification of the isolated fungal species

The developing fungal colonies were identified up to the species level by microscopic examination. This was made through the help of the following references: (Gilman, 1957), (Barron, 1972), (Samson and Reenen-Koekstra, 1988), (Moubasher, 1993) and (Kern and Blevins, 1997).

Cultivation of the isolated fungal species

The original fungal stock culture was subcultured on plates containing xylan agar medium and incubated at 25 ^{o}C for 2-4 days. Discs of 1cm diameter were cut from the 2-4 day old cultures used for inoculating the conical flasks which contained 100 ml of sterile liquid medium each. The flasks were incubated for 10 days at 25 ^{o}C static incubator. Than the culture was filtered with filter paper to separate the mycelium. The filtrate was treated for preparing xylanase enzymes.

Linear growth

Volumes of xylan agar medium were sterilized by autoclaving. Aliquots of about 15 ml of the medium were dispersed into sterile Petri-dishes, (9 cm diameter). Each dish was inoculated at its center with a fungal disc (1 cm diameter) cut from the colony margin of 2-4 day old cultures. The plates were incubated at 25 °C for 3, 5 and 7 days. Diameters of colonies were measured (mean of two diameters at right angles to each other) (Santiago *et al.*, 2006).

Total dry matter

Sterile xylan liquid media was dispensed to 250 ml Erlenmeyer flasks (100 ml per flask). Each flask was inoculated with a fungal disc (1 cm diameter) from the 2-4 day old cultures margin. Triplicate flasks were incubated at 25 ^{o}C for 10 days, filtered and the mycelium was washed several times with distilled water and dried between 2 filter papers. The mycelium was dried at 80 ^{o}C till the constant weight to get the dry weight expressed as g/100 ml growth medium (Aquino *et al.*, 2007).

Preparation of extracellular crude xylanase enzyme

Ammonium sulfate (80%) was added to the clear filtrate. The mixture was allowed to stand in cold temperature (5 ^{o}C) for 48 hours, and the precipitate was collected by centrifugation at 5000 revolution per minutes (r.p.m.) using SIGMA Laboratory Centrifuge for 20 min. The precipitate was dissolved in sodium citrate buffer (pH 7.0) and stirred for 30 minutes at room temperature. Elimination of excess salt was carried out by dialyzing the enzyme preparation overnight at 5 ^{o}C using sodium citrate buffer (pH 7.0) (El-Safey and Ammar, 2004).

Assay of xylanase enzyme

By using 0.01% birch wood xylan as substrate in 0.05 M sodium citrate buffer pH 7, xylanase was assayed. Xylanase activity was measured by incubating the reaction mixture (3.5 ml) which contained 1 ml of the enzyme solution with 1 ml of the substrate at 50 ^{o}C for 10 min incubation period. The tubes were put in boiling water both for 10 min then rapidly cooled. One halb ml phenol (10 ml phenol dissolved in 10 ml distilled water) was added to 1 ml of sulfuric acid and measured using UV-Spectrophotometer (UV-1650 pc, UV-Vis Spectrophotometer, Shimadzu) at 430 nm. A xylanase unit was defined as the release of one µmol of reducing sugar as a xylose per minute (Salama *et al.*, 2008).

Selection of the most efficient fungal species producing xylanase

All isolated fungal species were assayed for their xylanase activities on birch wood xylan substrate. The fungal species of the highest significant extracellular xylanase activity were selected and identified in the Regional center for Mycology and Biotechnology, Al-Azhar University, Egypt.

Optimization of the growth medium for the maximum production of extracellular xylanase by the selected fungal species

The components of xylan medium were substituted by

different sources. Triplicate flasks were used for each treatment. Xylanase activity was measured in the culture filtrates of the most efficient selected fungal species.

Nutritional factors

Effect of different carbon sources on the induction of xylanase

Carbon sources as:- glucose, xylan (control), sucrose and starch were used to prepare xylan medium on equal carbon basis. Triplicate flasks that contained xylan liquid media were substituted with different carbon sources, inoculated with efficient selected fungal discs and incubated at 25 ^{o}C for 10 days (Garg *et al.*, 2009).

Effect of different nitrogen sources on the induction of xylanase

The liquid medium that containing the best chosen carbon source from the previous experiment was used. Nitrogen sources were added:- inorganic nitrogen sources (ammonium nitrate and sodium nitrate), and organic nitrogen sources (peptone (control) and yeast extract) on equal nitrogen basis. The flasks were inoculated with the selected fungal discs and were incubated at 25 ^{o}C for 10 days. The culture filtrates were used to assay xylanase activity of the selected fungal species (Gupta *et al.*, 2009).

Physical factors

Effect of different pH values on the induction of xylanase

The medium containing the best sources of the nutritional factors. The following buffer solutions were used:- citrate buffer solution for acidic pHs (3 and 5) and boric acid-borax buffer solution for alkaline pHs (7 (control), 9 and 11), each at 0.05 M concentration. The flasks were inoculated with the selected discs and incubated at 25 ^{o}C for 10 days (Zhou *et al.*, 2009).

Effect of different incubation temperature values on the induction of xylanase

Triplicate flasks of liquid medium that contained the best source of chosen nutritional factors and the optimum pH were used. Incubation was at 10, 25 (control), 40, 55 and 70 ^{o}C, respectively for 10 days. The culture filtrates were used as xylanase enzyme sources for assay xylanase activities of the selected fungal species (Gupta *et al.*, 2009).

Ultraviolet radiation mutagenesis

Source of UV irradiation

The (UV) source was Philips TUV-30-W-245 nm Lamp, typ No. 57413-P/40 in King Abdul-Aziz University central lab at exposure dose 254 j/m^2 and exposure times 10, 20, 30, 40, 50, 60, 70 and 80 min.

Effect of UV radiation on the induction of xylanase

Soluble xylan media inoculated with selected fungal species were put inside UV cabinet at exposure dose 1800 j/m^2 at different times. All flasks were incubated for 10 day at 25 ^{0}C and the xylanase assay were measured in the culture filtrate by UV-Spectrophotometer (UV-1650 pc, UV-Vis Spectrophotometer, Shimadzu) at 430 nm (Santiago *et al.,* 2006).

Purification of extracellular xylanase enzyme produced by the selected fungal species

Crude extract

The crude extract was prepared by filtering the culture. The solution was rotated at 13000 r.p.m. to remove muddy matter. The protein content and the xylanase activity were assayed for this crude extract preparation.

Precipitation by salting out with ammonium sulfate

The xylanase solution was treated with ammonium sulfate by raising the concentration of ammonium sulfate to 80%. The precipitate formed was suspended in 0.05 M phosphate buffer (pH 9.0), centrifuged at 13000 r.p.m. for 20 min. This step was repeated at least three times to assure the complete elution of the co-precipitated enzyme (Weihua and Hongzhang, 2008) .

Dialysis

The precipitate was dissolved in 0.05 M phosphate buffer (pH 9.0) and placed in a dialysis bag and dialyzed against distilled water in a refrigerator for 48 hours, till solution was free from sulfate in the bag so we must chang distilled water outside the bag several times. Dialysis was repeated against sucrose for 48 hour, to elute excess of water. After dialysis, the protein content

and extracellular xylanase enzyme activity were determined (Salama *et al.*, 2008).

Application of extracellular xylanase enzyme on column chromatography

Equilibration

By using 0.05 M phosphate buffer (pH 9.0) and passed it through the column till the pH of the effluent became the same as the starting buffer.

Column packing

A column end was set up vertically and the column unit was fitted into the bottom of a column and tightened into a place. The slurry was gently stirred and poured into the column through a funnel. The effluent was allowed to run then the column end unit was inserted into the top of the extension tube. The flow rate should be greater than the desired elution flow rate at the end of the packing operation. The column was allowed to pack till the bed height attained a stable value (within 60 min). After packing, the extension tube was disconnected. The column was filled with buffer carefully to exclude any air bubbles. The top unit was reconnected and the bottom tap was opened. One bed volume of buffer was pumped through the column at the desired elution rate and absence of dead space between end unit and column bed was checked before loading with the enzyme solution.

Sample loading

The dialyzed sample was incorporated into the column for elution. The eluate was collected (5ml /fraction). The protein was determined. The xylanase activity was assayed and fractions were pooled for more investigation. Column chromatography of gel filtration chromatography (Sephadex G-100) and basic anion exchange (DEAE- Sephadex) were used (Salama *et al.*, 2008).

Sephadex G-100 Column

Phosphate buffer of pH 9.0 was used and the slurry was allowed to swell for 3 days at room temperature. Acolumn (2.5 × 39 cm) and Sephadex G-100 (SIGMA -S6147-25G) were used. The enzyme preparation sample was applied carefully to the top of the gel and was allowed to pass into the gel by running the column. Buffer was added without disturbing the gel surface. Fifty fractions (5 ml for each fraction) were collected. Enzyme activity and protein were carried out for each fraction. Fraction with peaks of xylanase were collected and pooled for the next step (El-Safey and Ammar, 2004).

DEAE-Sephadex Column

The gel was soaked in 0.05 M phosphate buffer pH 9.0 for 72 hour. The suspension was well mixed and poured at once down the column. The pooled active fractions were dialyzed against sucrose using dialysis bag and was applied in small amount of buffer to DEAE-Sephadex column (2.5 × 39 cm). The enzyme was eluted to DEAE-Sephadex column in a small amount of buffer in succession with 0.05 M phosphate buffer of pH 9.0, 0.05 M NaCl in 0.05 M phosphate buffer of pH 9.0 (100 ml), 0.1M NaCl in 0.05 M phosphate buffer of pH 9.0 (100 ml) , 0.2 M NaCl in 0.05 M phosphate buffer of pH 9.0 (100 ml) and 0.3 M NaCl in 0.05 M phosphate buffer of pH 9.0 (100 ml). The enzyme was eluted with the same buffer and 5 ml fraction was collected (Salama *et al.*, 2008).

Protein determination

Protein concentration was determined by Biuret test (copper sulfate solution, potassium sodium tartarate and sodium hydroxide) with bovine serum albumin as standard (Eakpetch *et al.*, 2008).

Test for the purity of the purified extracellular xylanase

Two criteria for the purity were testimonized. The first one was DEAE-Sephadex column chromatography (final stage of purification). The second was given by applying sodium dodecyl-sulphate-polyacrylamide gel electrophoesis (SDS-PAGE). The SDS-PAGE system with homogenous gel was carried out at the King Fahd Medical Research Center according to Laemmli (1970). Here are some solutions required for the preparation of gel:-

Acrylamide stock solution 30% (w/v) acrylamide, 0.8% (w/v) methylene bisacryl-amide, Resolving gel buffer (pH 8.8), Stacking gel buffer (pH 6.8), (SDS) solution 10% (w/v) SDS, Ammonium persulphate (APS), N,N,N',N',-tetramethylethyen-diamine (TMED), Sample buffer (5-fold concentrated), Electrode buffer (pH 8.8).

Normally, resolving gel with 12.5% acrylamide was used with such constituts :- (a) Acrylamide stock solution , (b) Stacking gel buffer, (c) SDS-solution (10%), (d) Water (dist.), (e) TMED and (f) APS. Stacking gel contained 4% acrylamide according to the preceding too.

Casting gels

Casting apparatus was assembled vertically. Resolving

gel components (a-d) were mixed and deaerated by vacuum and the components of (e -f) were mixed gently. The resolving gel was poured immediately into the space between the glass plates to the desired height, overlaid gently with water and was left to polymerize. Water layers were removed. The stacking gel components (a-d) were mixed and deaerated, then components of (e -f) were mixed gently. A comb was inserted carefully between the glass plates then removed after polymerization. The plates were fixed in the electrophoresis tank and we filled the electrode buffer. A bent syringe needle removed the air bubbles beneath the gel.

Sample preparation

A protein sample was mixed with 3 ml of sample buffer and heated in a boiling water bath for 5 min then centrifugation at 14000 r.p.m. for 5 min at 4 ^{o}C. The protein samples were loaded using wells in stacking gel under the electrode buffer using microliter syringe.

Electrophoresis run conditions

It is carried out at 4 ^{o}C under constant current, then the dye had reached the bottom of the gel, the electrophoresis was stopped and the plates were removed from the tank. The inner and outer glass plates were separated and the gel was detached and subjected to staining in 50 ml of 0.1% commassie blue, 40% methanol, 10% glacial acetic acid. The gel was destained in distaining solution (10% glacial acetic acid and 40% methanol), gel was sandwiched with cellophane membrane and dried on gel drier for 2 hour and photographed.

Characterization of the purified extracellular xylanase enzyme

Reaction temperature

Identical reaction mixtures were incubated at the following temperatures: 30, 40, 50, 60, 70 and 80 °C for 10 min. Control was made using denatured inactivated enzyme instead of the active. Xylanase activities were assayed (Lee *et al.*, 2009).

Thermostability

The purified xylanase solutions were allowed to stand in water baths at various temperatures (40, 50, 60, 70, 80 and 90 ^{o}C) for one hour. The heat-treated solutions were rapidly cooled and xylanase activities were assayed (Salama *et al.*, 2008).

pH value

The reaction mixture was carried out with the substrate and pure enzyme using the buffer system with different pH values (7-12). Incubation was conducted at the optimum temperature for 10 min. Xylanase activity was assayed (Fang *et al.*, 2008).

RESULTS AND DISCUSSIONS

The present work deals with physiological and biochemical studies on extracellular fungal xylanase enzymes. It included studies on the productivity of alkalo-thermophilic xylanases by different fungal cultures. Optimization of culture conditions for maximum xylanases productivity was carried out. Radiation mutagenesis by using ultraviolet radiation for stimulating the production of extracellular xylanase produced by the selected alkalo-thermophilic fungal species was recorded. The research will also include the purification and characterization of the pure alkalo-thermophilic xylanase produced by the potent fungal strain.

In the present study, the isolation of fungal species was carried out from seven soil samples collected from Makkah road (Makkah high way 10 Km east of Jeddah district and 40 Km south of Jeddah district), Al-Madina Al-Monawara road [Al-Madina high way, (30 Km north of Jeddah district) and Obhur way (50 Km north of Jeddah district] and three agriculture soil sites (20 Km east of high way), [*Ocimum basilicum* (Ocimum) agriculture soil, *Ziziphus spina-christi* (Ziziphus) agriculture soil and *Phonix canariensis* (Date Palm) agriculture soil] located in Jeddah district, Saudi Arabia. A total of 188 fungal colonies were captured from all seven soil samples.Ten fungal species *(Alternaria alternata, Aspergillus candidus, A. flavus, A. ochrochus, Botrytis cinerea, Fusarium roseum, Penicillium canescens, P. chrysogenum, P. italicum* and *Rhizopus microsporus*) related to six genera were isolated from the seven tested soil samples. *Aspergillus flavus* was isolated with the highest occurrence and population density (67 isolates representing 35.63%). Moderate occurrence was showed by both *Penicillium chyrsogenum* and *Rhizopus microsporus* with relative densities of 21.27 (14.89%). The rest fungal species were collected with low and rare occurrence. It seems that variation in fungal count is probably due to the ability to metabolize the different decomposition products in the tested soil samples The densest tested fungal soil (106 colonies) was agriculture soil sites, while Al-Madina Al-Monawara road was recorded as the least counting fungal site (39 colonies) (Table 1).

The linear growth, dry weight gain and xylanase productivity of all isolated ten fungal species were

Table 1. Total counts and numbers of isolated fungal species all over the tested seven soil samples located in three different sites in Jeddah district, Saudi Arabia.

Fungal species	Total colonies count/ g dry soil	Realative density %	Frequency of occurrence
Alternaria alternata	9.0	4.79	L
Aspergillus candidus	8.0	4.26	R
A. flavus	67.0	35.63	H
A. ochrochus	10.0	5.32	R
Total Aspergilli	85.0	45.21	-
Botrytis cinerea	8.0	4.26	R
Fusarium roseum	8.0	4.26	L
Penicillium chrsogenum	40.0	21.27	M
P. italicum	8.0	4.26	L
P. canescens	2.0	1.06	R
Total Penicilli	50.0	26.59	-
Rhizopus microsporus	28.0	14.89	M
Total count	188.0	100.0	-
Total number of species	10.0	-	-

Table 2. Assay of extracellular xylanase enzyme activities for the isolated ten fungal species.

Fungal species	Xylanase activity (unit/ml)
Alternaria alternate	0.27
Aspergillus candidus	0.60*
A. flavus	0.29
A. ochrochus	0.51
Botrytis cinerea	0.47
Fusarium roseum	0.28
Penicillium canescens	0.22
P. chrysogenum	0.29
P. italicum	0.43
Rhizopus microspores	0.16
LSD at 0.05	0.04

* High significance

determined. *Fusarium roseum* followed by *Penicillium chrysogenum* gave the highest linear growth (8.2 and 6.7 cm), while *Penicillium chrysogenum* has the highest significant dry weight gain (0.27 g/100 ml). An inverse relationship was existed between the induction of the tested extracellular xylanase enzyme activity and the mycelial dry weight, where the highest significant xylanase activity was shown in *Aspergillus candidus* (0.60 unit/ml) was coupled with the lower dry weight gain (0.11 g/100 ml). According to statistical analysis of the present experimental data, *A. candidus* was selected as the most efficient producer for extracellular xylanase (Table 2).

Optimization of the culture conditions for the highest xylanase producing species (*Aspergillus candidus*) was carried out to improve xylanase production. The suitability of the nutritional requirements (carbon and nitrogen sources) and environmental conditions (pH and temperature) on the productivity of xylanase enzyme were investigated. The following different carbon sources [glucose, xylan (control), sucrose and starch] was applied. The highest significant value of *A. candidus* extracellular xylanase (0.60 unit/ml) occurred on xylan carbon source and this may be due to the inducible nature of xylanase enzyme. In the present investigation, a basal medium inducible xylan as a best carbon source

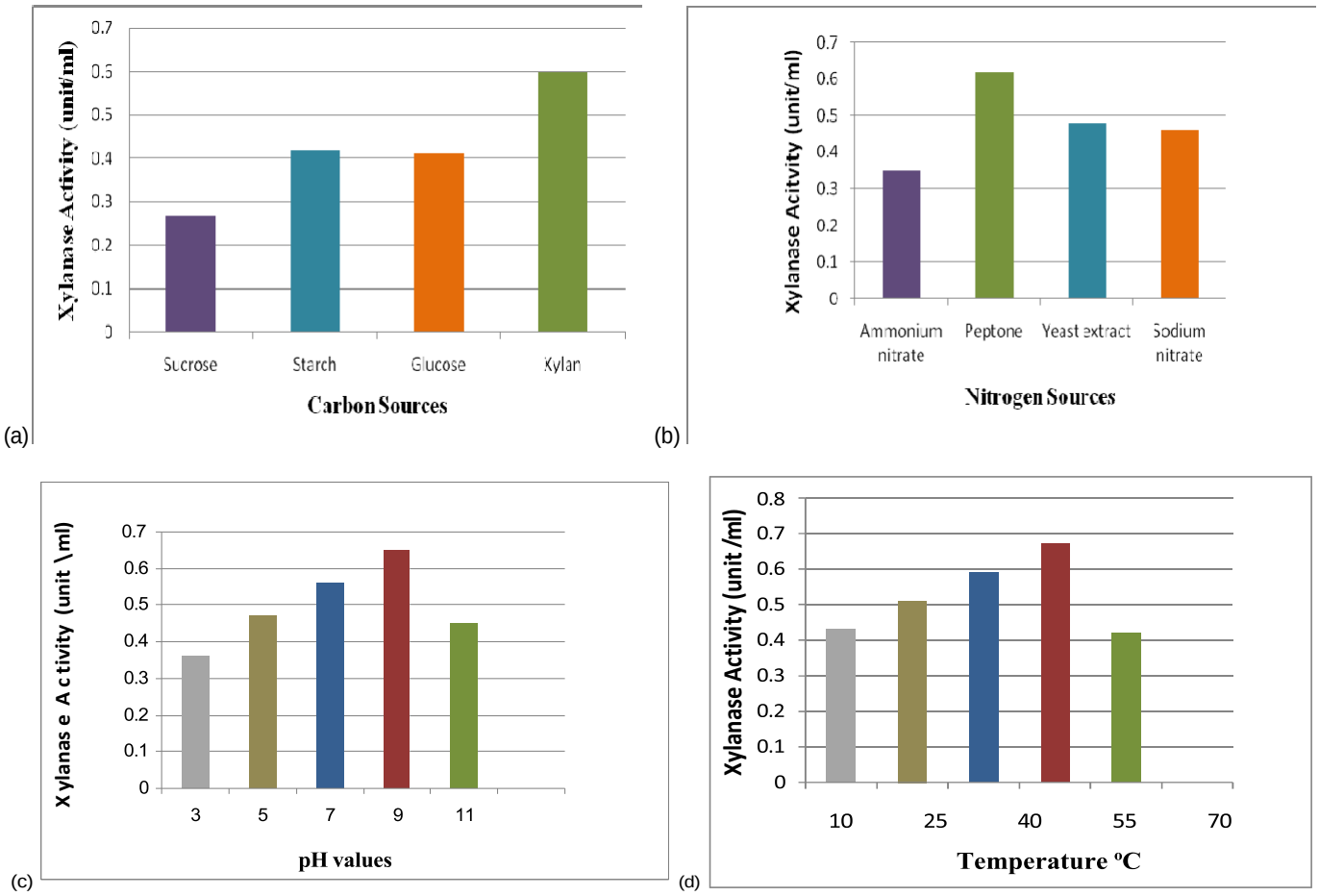

Figure 1. Optimization of the cultural conditions to improve xylanase activity of *A. candidus* - effect of different (carbon and nitrogen sources) and (pH and temperature values**)** on xylanase enzyme activity (unit/ml) produced by *A. candidus* after 10 days incubation period at 25 ºC

for *A. candidus* xylanase productivity was modified by adding different sources of nitrogen to evaluate the effect of these sources of nitrogen on xylanase production and activity. Maximum significant value of xylanase activity (0.62) was manifested with peptone as organic nitrogen source.

Alkaline pH value (9) had a highest significant effect (0.65 unit/ml) on the productivity of extracellular xylanase by *A. candidus*. The increase in xylanase activity at alkaline pH may be refer to the fact that respiration by cells of alkalophilic fungi increased with increase in the external pH values and was maximum at pH 9 (Geweely, 1997). Alkalophilic fungi have got enzymatic systems that enable them to utilize nitrogen and the synthesis of protein becomes active in an alkaline environment (Esmat and Geweely, 1999). It is known that the pH of the medium affects the availability of certain metallic ions, membranes permeability and enzymatic activities. The increase in the tested xylanase activity by *A. candidus* at alkaline pH (9) may be attributed to the fact that the ribosomes of alkalophilic microbe contributed to the higher activity of protein synthesis at alkaline pH (Ellil and Geweely, 1999). The decrease or increase in enzyme activities at different pH values than the optimum pH might be due to decreased production of the mycolytic enzymes (Sarao *et al*., 2010). Study on optimization of the tested *A. candidus* xylanase by different temperature values was performed at temperature range from 10 to 70 °C. The optimum temperature for the highest significant production of extracellular *A. candidus* xylanase (0.67 unit/ml) was at 55 °C. The decrease in activity of xylanase at very low and high temperature compared to 55 °C might be due to the slow growth at low temperature and inactivation of the enzyme at high temperature (Figure 1 (a.b.c.d).

The productivity of xylanase by *A. candidus* was more sensitive with increasing exposure time of ultraviolet radiation. Radiation mutagenesis was occurred on the

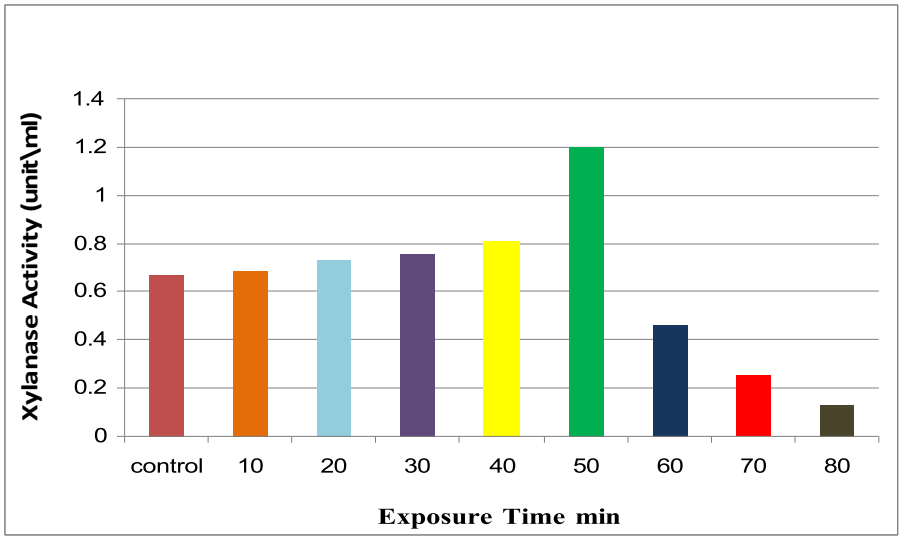

Figure 2. Radiation mutagenesis of xylanase enzyme produced by *A. candidus* -Effect of ultraviolet radiation at different exposure times on xylanase enzyme activity (unit/ml).

Table 3. Purification scheme of extracellular xylanase produced by irradiated *Aspergillus candidus*.

Purification steps	Total Volume (ml)	Total Activity (unit/ml)	Total Protein (mg)	Specific activity (unit/mg)	Purification Fold	Recovery (%)
Crude enzyme	100	0.686	0.395	1.736	1.000	100.0
Ammonium Sulfate	60	0.665	0.375	1.773	1.021	96.9
Dialysis	30	0.641	0.331	1.936	1.115	93.4
Sephadex G-100	2					
Peak A		0.630	0.283	2.226	1.282	92.0
Peak B		0.630	0.319	1.975	1.137	91.8
DEAE Sephadex	2	0.621	0.275	2.258	1.301	90.5

tested *A. candidus* and enhanced xylanase productivity after 50 min exposure time, where the maximum significant production of xylanase (1.20 unit/ml). The radiation may also stimulate and mutat the gene responsible for xylanase production. Our finding is coupled with that recorded by Geweely *et al*., (2006) who stated that the total protein, total activity and specific activity were significantly higher in xylanase recovered from the irradiated *Aspergillus terreus* than the non-irradiated fungus (Figure 2).

Investigation the methods involving the production of pure homogenous extracellular irradiated *A. candidus* xylanase was carried out by using ammonium sulphate precipitation and dialyses to concentrate the xylanase activity and chromatography by combination of gel filtration and basic anion exchange DEAE- Sephadex to purify the enzyme preparation. The highest precipitation was obtained by 80% ammonium sulphate followed by dialyses against distilled water and then against sucrose to concentrate the activity of the crude extract of extracellular xylanase, where the specific activity raised to 1.115 fold over the crude xylanase extract. This increase in activity is presumably by removal of low molecular weight inhibitors, possibly phenolic substance released from the growth substrate. Purification of the tested extracellular *A. candidus* irradiated xylanase on Sephadex G-100 separated two peaks of xylanase (A and B) with 1.282 and 1.137 fold increase over the crude extract, respectively (Table 3, Figure 3). This increase in purification fold may indicated a high xylanase content in the original fungal extract relative to other proteinaceous compounds. This may be expected because the selected

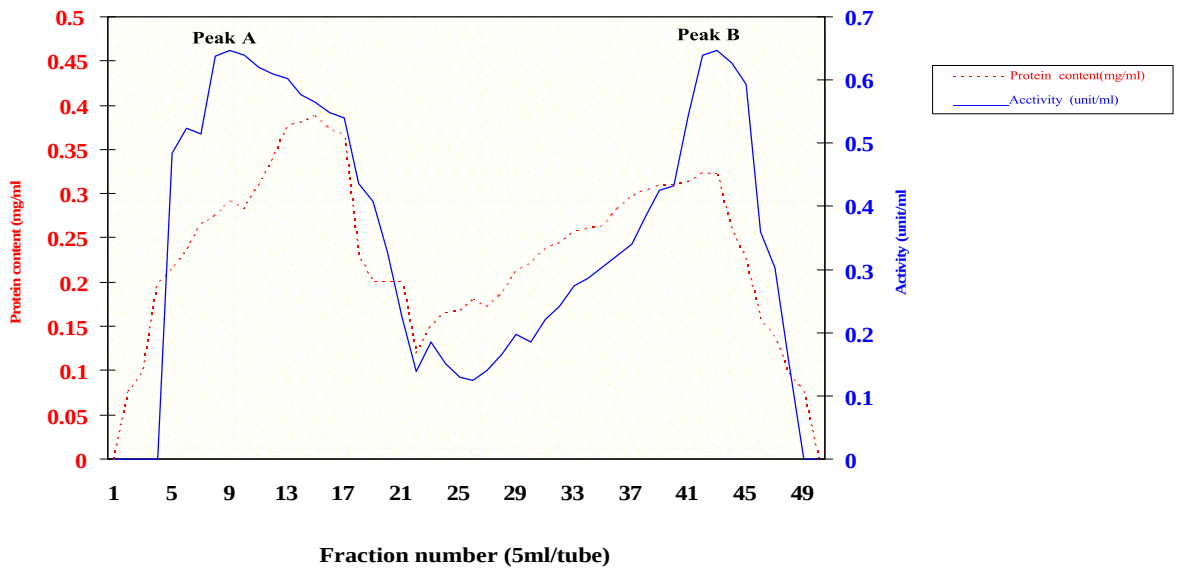

Figure 3. Typical elution profile for the behavior of extracellular xylanase produced by irradiated *A. candidus* on Sephadex G-100.

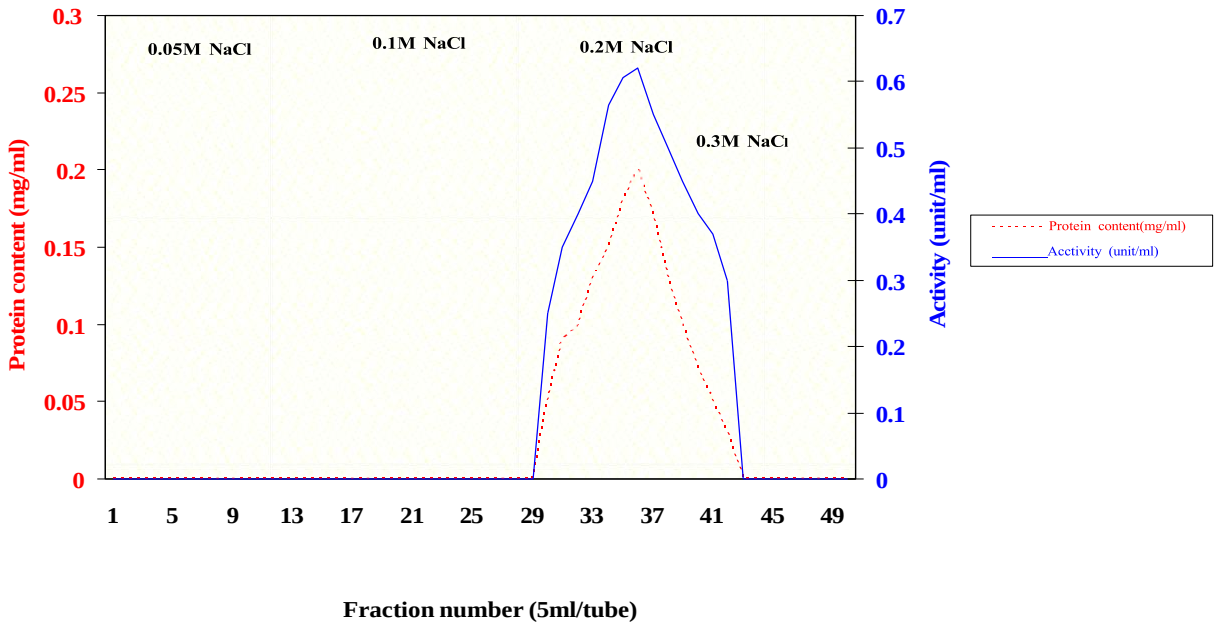

Figure 4. Typical elution profile for the behavior of extracellular xylanase produced by irradiated *A. candidus* on DEAE –Sephadex.

fungal species have high xylanase activity. Each of the active xylanase fractions were pooled, concentrated and applied to DEAE-Sephadex column. From the elution profile on DEAE-sephadex column, it can be seen that a single purified peak of *A. candidus* xylanase followed by one peak of protein was obtained with increase in the specific activity (2.258 unit/mg), which represent 1.301 folds of purification over the crude extract (Figure 4).

Test for the purity of the tested purified *A. candidus* extracellular irradiated xylanase was detected in the present investigation. Two criteria for the purity of the purified *A. candidus* extracellular xylanase were

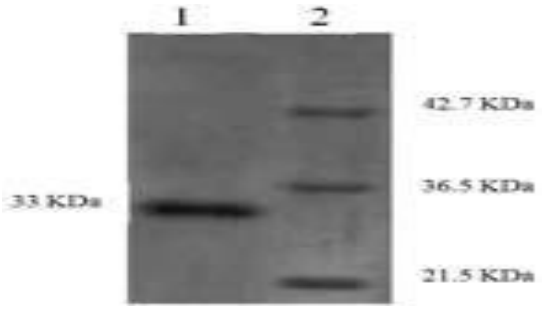

Figure 5. SDS-PAGE analysis of purified xylanase. Lane 1: purified xylanase; lane 2: molecular mass marker.

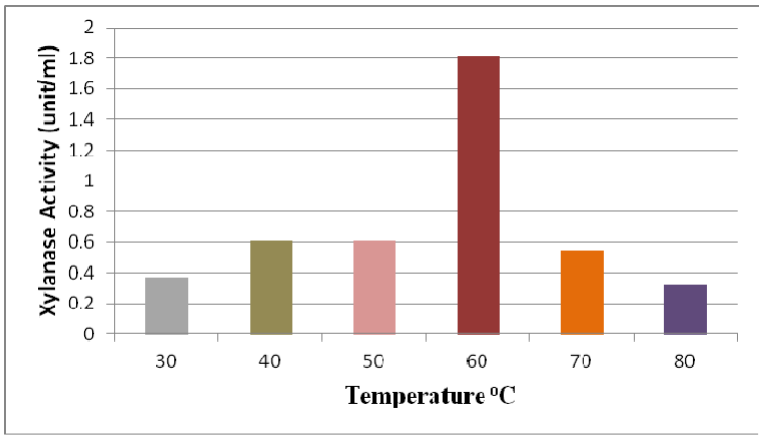

Figure 6. Effect of temperature values on the irradiated purified xylanase enzyme activity produced by *A. candidus*.

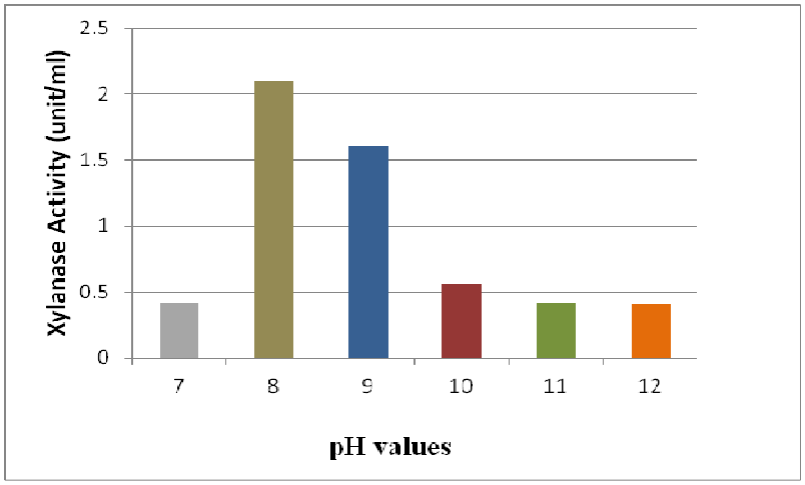

Figure 7. Effect of different pH values on the activity of the irradiated purified xylanase enzyme produced by *A. candidus*.

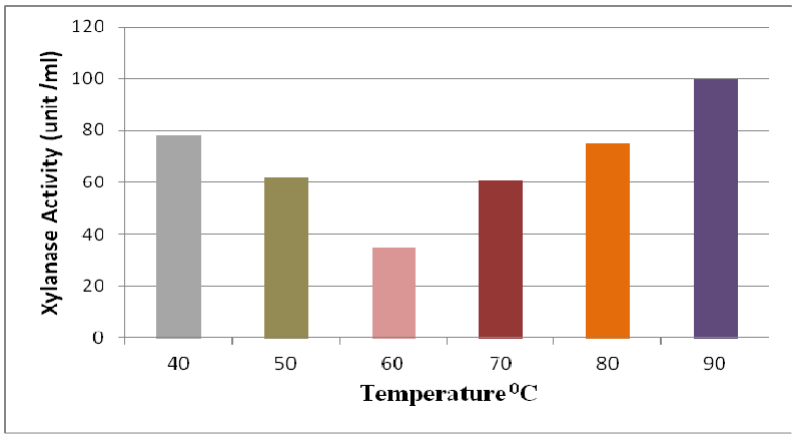

Figure 8. Thermostability of the irradiated purified *A. candidus* extracellular xylanase after 60 minutes incubation time. Loss of activity (%) was determined under standard assay conditions.

testimonized. DEAE-Sephadex column chromatography (final stage of purification) resulted in a single sharp peak of *A.candidus* extracellular xylanase. The second criterion was given by applying SDS-PAGE electrophoresis technique. One single pure xylanase protein spot stained with commassie blue was observed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 5).

Characterization of the irrdiated purified extracellular xylanase enzyme in the present study revealed that the optima reaction temperature (1.82 unit/ml) and pH (2.10 unit/ml) for the irradiated purified xylanase were 60°C and 8, respectively (Figure 6, 7). Thermostability of the purified *A. candidus* xylanases showed that a complete inactivation of xylanase enzyme was attained at 90 ^{o}C after 60 minutes exposure time. The minimum significant loss of xylanase activity (35%) was detected at 60 °C. This result suggests that thermostability of xylanase may be due to the presence of disulfide bonds which lead to more heat stability. Lee *et al.* (2009) stated that two disulfide bridges are formed by xylanase leading to thermostability (Figure 8).

CONCLUSIONS AND RECOMMENDATION

In conclusion, the use of ultraviolet radiation is a promising technology for stimulation of xylanase enzyme which is very important in biotechnology application field helping in the pretreatment of paper pulps to reduce the dependence on chlorine used for bleaching in brightening process, animal feed, pharmaceutical industries, production of xylitol to be used as sweetener food additive in many food products, chemical industries and retting of fibers in industries applications. The use of the irradiated purified alkalo-thermophilic extracellular xylanase isolated from *A. candidus* which captured from Saudi Arabian soil was preferable enzyme in industrial field and nano technology to produce a large amount of the enzyme at the lowest cost, rather than the use of high expensive chemicals, where the most advantageous working conditions prevailing for application of xylanase in biotechnology at high temperature and alkali conditions. Therefore, an ideal xylanase should be able to function under the extreme conditions.

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