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Effect of Different Agricultural Wastes on Xylanase Production by *Saccharomyces cerevisiae and* its Application on Citrus Fruit

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Agricultural wastes are low cost, renewable and most abundant raw materials present in environment. Different agricultural wastes were tested for xylanase production from *Saccharomyces cerevisiae.* Mixed culture of soy bean and wheat bran (4:6) was most effective against pathogenic fungi and suitable for maximum xylanase production at pH 5.0 after 48 h incubation period. Xylanase was stable at 55°C for 20 min. The molecular weight of crude xylanase was 74 KDa. Coating of citrus fruits with *S.cerevisiae* grown on mixture of wheat bran and soybean was highly significant reduction of both disease incidence and disease severity. Xylanase can be used as biocontrol for green and blue mold of orange and lime fruit.

Keywords: Xylanase production, *Saccharomyces cerevisiae*, citrus fruit, green mold- *blue mold*.

Xylanase (E.C.2.8.1.8) defined as a group of hemicellulose which required for the hydrolysis of 1,4-xylans present in lignocellulostic materials[1].

Xylanase plays an important role in nature which being a part of protecting environment from pollution because of their alternative way to chemical hydrolysis .The industrial applications of xylanase came from their ability to hydrolyze xylan which are abundant natural polysaccharide[2].

Xylanase from different microorganisms such as fungi , bacteria and few yeast strains gained interest due to their potential applications in many industrial processes such as production of hydrolysates from agro-industrial[1],nutritional improvement of lignocellulosic feed stuff[3], clarification of wines and juices and biobleaching of craft pulp in paper industry[4,5,6] .

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Raw material such as sugar cane bagasse, wheat bran, rice bran and corn cobs are agricultural wastes can also be used for developing biotechnological process of industrial interest[7]. Plant cell walls contain 20-40% xylan biomass as major component. Therfore hydrolysis of xylan is important for utilization of lignocellulosic material in nature[2].

Post-harvest decay of citrus fruit caused by *Penicillium digitatum* Pers. Sac (green mold), *Penicillium italicum* Whemer (Blue mold) have been reported all over the world and represents major losses in production, during harvest, storage and exportation[8].

In Egypt, losses of about 10% and 15% of orange and lemons in storage, respectively, occur each year[9, 10].

The main object of this study was evaluation of various agricultural wastes under optimum conditions for maximum yield of xylanase from *Saccharomyces cerevisiae* in liquid culture medium and its applications on post-harvest diseases.

MATERIALS AND METHODS

Microorganism

The antagonistic yeast used was *Saccharomyces cerevisiae*[Meyen ex E.C.Hansen] were obtained from Plant Pathology Department of National Research Centre, Giza, Egypt. The strain was kept on potato dextrose liquid medium and stored at 4°C.

Substrates

Different agricultural wastes (corn cobs, cotton straw, wheat straw, shrimp shell, rice straw, soy bean, wheat bran, pea peels and chitin) were used as sole carbon source and tested for xylanase production. All wastes were washed, dried at 70°C in oven and cut into small pieces before use.

Fermentation media

The ability of the microorganism for xylanase production was examined in media containing (g/l):$NaNO_3$ (2.0) , $MgSO_4$ (0.5) , $CaCO_3$ (3.0) , different agricultural wastes (20.0). Erlenmeyer flasks 250ml each containing 50 ml fermentation media was inoculated with 1 ml of *Saccharomyces cerevisiae* and incubated at 28-30°C for 48 h. in incubator shaker at 200 r.p.m.

Xylanase assay

Determination of enzyme activity was carried out according to the method of[11]:

One ml of 1% birch wood xylan (Sigma) in acetate buffer (pH 4.6) in test tubes was added to one ml of the culture filtrate and mixed by shaking.

The mixture was incubated in a water bath at 50°C for 30 minutes, then cooled and centrifuged before assaying. The amount of reducing sugar was determined with 1 ml of 3, 5-dinitrosalicylic acid (DNS) . Unit of enzyme: one unit of enzyme activity was taken of the catalyst convert one micromole of substrate in one minute under specific condition.

Optimization of culture conditions

Culture conditions were optimized at different ratios of agricultural wastes, different pH values and thermal stability.

In vitro: Evaluation of antifungal activity of *S. cerevisiae* grown on different agricultural wastes

Saccharomyces cerevisiae grown on different agricultural wastes was screened for their antifungal activity (inhibit mycelium growth) using the well-plate diffusion method as described by[12]. Pathogenic fungi over laid on PDA plate and after 30 min. three wells (5mm. in diameter) were made in each plate and inoculate with 20ml of extracts in contrast with the same volume of *S. cerevisiae* free and control (water). The plates were kept for 2h.at 4°C to allow diffusion of the extract then incubated at 28°C for 5days. Diameter of inhibition zone was measured.

Management of green and blue molds disease of orange and lime fruits during storage

Different treatment of *S. cerevisiae* formulated on wheat bran or soybean alone or in combination wheat bran: soybean(4:6) were tested to study their efficiency on management of green and blue molds incidence on orange and lime fruits during storage.

The promising treatment of as follows:

1.*S.cerevisiae* concentration (10^6 spores/ml).
2.Wheat bran
3. Soy bean
4.*S. cerevisiae*+wheat bran
5. *S. cerevisiae* +soybean,
6. *S.cerevisiae* + wheat bran : soybean (4:6)
7. Control (untreated inoculated fruits).

Commercially harvested navel oranges (*Citrus sinensis*L. Osbeck) and lime (*Citrus aurantifolia* F. Muell), with healthy appearance from citrus orchards were used in this investigation. A highly aggressive isolates of *Penicillium digitatum* and *Penicillium italicum* originally isolated from rotted citrus fruit .

Isolates were grown on potato dextrose agar (PDA) at 25°C for 7 days. Spore suspension (10^6 spores / ml)was obtained by flooding 7thday old cultures of pathogen with sterile distilled water containing 0.01% (v/ v) Tween 80[13].

Fresh orange and lime fruits apparently free from physical damage and diseases were artificially wounded using sterilized scalpel. Inoculation of wounded fruits was carried out by spraying fruits with spore suspension of *P. digitatum* and or *P. italicum* then air dried at room temperature. Inoculated fruits were dipped for 4 min.in solution of treatment and untreated (control) orange and lime ,after air dried placed into carton boxes at rate of 15 fruits /box of orange and/or lime fruits respectively. Each particular treatment as well as control treatment was represented by five carton five boxes. All boxes were stored at 20°C for 20 days.

Statistical analysis

Statistical analyses were performed with descriptive statistics (mean) and inferential tests (ANOVA followed by Tukey test) to determine statistically significant differences ($P < 0.05$) between treatments with Sigma Stat Software 2013[14].

Gel electrophoresis of crude xylanase

Lysates prepared from control and infected trypsin treated and untreated cell lines were analyzed by SDS-PAGE according to previously mentioned by[15]. Lysates were separated through 4% stacking and 12.5 % resolving gels in a Bio-Rad Mini-Protean II electrophoresis chamber (Bio-Rad Laboratories, Munich, Germany). Diluted samples (1:1) in reducing sample buffer were heated in boiling water bath for 5 min then loaded onto the stacking gel (20 µl of each sample/ each lane). To ensure allow appropriate determination of the molecular weights of the resolved protein fractions, low range molecular weight marker protein was loaded on the same gel. Electrophoresis was performed at 80 m/V constant voltage and stopped after the bromo-phenol blue dye reached the end of the resolving gel. After electrophoresis, gels were stained using coomassie and silver staining. The gel was soaked in the coomassie staining solution at 37°C for 2 h. in a shaker incubator. After staining, the gel was washed twice with H_2O to remove excess stain then covered with distaining solution. Three to four changes of distaining solution was usually sufficient to visualize blue bands in a clear background.

RESULTS AND DISCUSSION

Xylanase production by *S. cerevisiae* from different agricultural wastes at various incubation time

Various agricultural wastes (corn cobs, cotton straw, wheat straw, shrimp shell, rice straw, soy bean, wheat bran, pea peels and chitin) were used as cheap and most economic carbon source for xylanase production from *S. cerevisiae*. Fermentation condition tested with different time intervals ranging from (24,48 &72)h. Results in Fig. (1) showed that soy bean and wheat bran produce maximum xylanase activity 51.4 &53.2 U/ml respectively after 48 h from incubation. The highest xylanase activity from *S. cerevisiae* was observed in pretreated rice straw than that of corn cobs as discussed by[16].The use of agricultural wastes as sole carbon sources reduces the production costs of the final products of *Saccharomyces* sp. and has the potential to produce xylanase from crude substrate wheat bran and bagasse[17].

Different ratios of wheat bran and soy bean

Ten different proportion ratio of mixture of wheat bran and soybean were tested for their ability for xylanase production by *S.cerevisiae* in comparison with control wheat bran and soy bean alone. Results in Table (1) showed that wheat bran: soybean (4:6) produce maximum xylanase activity 91.7 U/ml followed by wheat bran: soybean (3:7) produce 66.0 U/ml , above and below this ratios moderate to low xylanase production was recorded. Production of xylanase by *Saccharomyces sp.*CD3 increased while using wheat bran and sugar cane bagasse as substrate[18]. Xylanase activity from *Aspergillus niger* and *Aspergillus ochraceus* using mixture of corn cobs and wheat bran increased18 % these results were reported by[19].

Different pH values on xylanase activity

Different pH values ranging from (3.6-7.0) were studied for xylanase production using wheat bran: soybean (4:6) as substrate .Results illustrated in Fig. (2)showed that maximum xylanase production was obtained when the initial pH of the medium adjusted at pH 5.0 produce 92.0 U/ml using the tested organism followed by pH 4.5 then pH 4.0. When the medium pH was elevated to 7.0 xylanase production gradually decreased, pH 6.0 determined in Mcllvaine's buffer produced by a

Table 1. Effect of different ratio of wheat bran and soy bean on xylanase production by *S. cerevisiae*

Wheat bran: Soybean	Xylanase activity (U/ml)
1:9	46.2
2:8	54.2
3:7	66.0
4:6	91.7
5:5	60.7
6:4	48.7
7:3	48.3
8:2	44.5
9:1	39.6
1:1	48.3
Wheat bran	53.2
Soy bean	51.4

Table 2. Effect of thermal stability on xylanase production by *S. cerevisiae*

Temp°C	Xylanase activity (U/ml)						
min	10	20	30	45	50	55	60
10	50.3	50.3	50.3	50.3	50.3	50.3	50.3
15	55.4	60.3	60.7	61.3	63.3	70.4	75.6
20	75.7	77.1	78.8	77.8	78.2	78.8	79.9
25	80.9	81	82.2	83.3	83.9	84.3	85.1
30	88.9	88.7	88.5	89.1	89.8	91.8	92
35	93.6	94	94	95.4	96.8	97.8	98.9
40	110.6	112.6	114.7	117.8	118.2	119.4	119.4
45	122.3	125.4	128.6	132	132	132	132
50	132	132	132	132	132	132	132
55	132	132	131.8	130.7	128.5	132	126.1
60	125	120	114	110	98	95	90

Table 3. Effect of *S. cerevisiae* grown on different agricultural waste at different incubation period against some pathogenic fungi

Different agricultural waste	Zone of inhibition (mm.)											
	P.digitatum			*P.italicum*			*Alternaria tenuis*			*Botrytis cinerea*		
Time/h.	24	48	72	24	48	72	24	48	72	24	48	72
Chitin	0	31	0	30	34.3	0	30	37.7	0	0	31	0
Pea	0	30	30	32.3	36	0	20.7	34.3	0	0	27	0
Corn cobs	32.7	34	0	37.7	30	0	30	35.7	0	37.6	33.3	0
Cotton stalk	32.7	35.3	0	31	35.7	0	21	33.7	0	26	25.7	20
Wheat straw	29.3	36	0	23	0	0	21.7	25.3	0	25.3	36.7	0
Shrimp shell	24.3	38.3	0	27	44	0	27.7	42.7	0	44	35.3	36.7
Saw dust	0	17.7	0	32.7	22.3	0	26	37.7	0	30.7	33.3	24.3
Rice straw	0	31	0	21.7	31.7	0	29.7	37.3	0	0	22.3	0
Soybean	35	37.7	0	40.7	42.3	25	37.7	47.7	21.7	31.7	49	27.7
Wheat bran	39	37.7	27.7	25	34	30	22.3	32.7	0	24.7	40	27.7
S.cerviceae	20	25	32	22	28	25	25	30	0	23	28	0
Control	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. Reduction % of green and blue molds on orange fruits in response to protecting effect of *S. cerevisiae* grown on wheat bran or soybean alone or in combination during storage at 20˚C after 20days

	Reduction %							
	Green mold				Blue mold			
Treatments	D.I.	R.	D.S.	R.	D.I.	R.	D.S.	R.
S. cerviceae	19.3b	70.7	15.0b	75	15.0b	80.8	13.3b	86.7
Soybean	0.0c	100	0.0c	100	0.0c	100	0.0c	100
Wheat bran	16.7b	73.3	3.3c	86.7	10.0b	87.2	10.0b	90
S. cerviceae +Wheat bran +soybean	0.0c	100	0.0c	100	0.0c	100	0.0c	100
Control	90.0a	-	100a	-	78.3a	-	100a	—-

Data in each column with the same letter are not significantly difference (P=0.05) according to Tukey test [14].
D.I. :Disease incidence , D.S. :Disease severity, R. : Reduction

Table 5. Reduction % of green and blue molds on Lime fruits in response To protecting effect of *S. cerevisiae* grown on wheat bran or soybean alone or in combination during storage at 20?C after 20days

	Reduction %							
	Green mold				Blue mold			
Treatments	D.I.	R.	D.S.	R.	D.I.	R.	D.S.	R.
S. cerevisiae	31.3b	68.7	17.5c	82.5	37.5b	52.1	15.5b	84.5
Soybean	75	28.3b	72.5	21.3c	72.8	18.7b	81.3	25.0c
Wheat bran	13.8d	72.5	13.8d	71.7	13.5d	82.4	6.7c	93.3
S. cerevisiae +Wheat bran + soybean	12.5d	86.2	6.7e	93.3	12.5d	84	5.0c	95
Control	100a	-	100a	-	78.3a	-	100a	-

Data in each column with the same letter are not significantly difference (P=0.05) according to Tukey test [14]

yeast strain called EBy 100-pYD1 was optimum for xylanase production[20] while xylanase largely obtained from yeast strain identified as *Cryptococcus lauren* was at pH ranged from 6.0 to 7.2[21].

Thermal stability

Thermal stability of xylanase was determined by incubating the enzymes on different temperatures 10-60°C under varying times .Results in Table (2)showed that xylanase activity increased till reached 50°C , and 55°C for 20 min. Then activity begin to decrease, while at 60°C xylanase loss its activity. Thermal stability at high temperatures above 40°C make enzyme more attractive for biotechnological applications in many industrial areas [22]. Enhances thermal stability of yeast makes enzymes more stable for industrial applications[23]

In vitro: Evaluation of antifungal activity of *S. cerevisiae* grown on different agricultural wastes

Saccharomyces cerevisiae grown on different wastes at different incubation period were studied their inhibitory effect on zone of inhibition of some pathogenic fungi.

Results in Table (3) indicated that *S.cerevisiae* grown on soybean or wheat bran was most effective against all pathogenic fungi compared with other waste as well as control and *S.cerevisiae* alone. The most effective treatment was after 48hr. incubation period. Different agricultural waste as baggases were used by[24] and recorded that boiled rice bran increased the growth of biocontrol agents .Major research on biocontrol is centered with the use of different agriculture waste to formulated bioagents.

The use of antagonistic yeasts in the control of grey rot were recommended to reduce and replace chemical fungicides[25,26] and sour rot[27] in grapes. Most of the reports dealing with biocontrol mechanisms depend on using single bio-fungicide yeasts or suppression of fungal diseases by single mechanism[28,29,30].

In vivo : Effect of orange fruits coating with *S. cerevisiae* formulated on soy bean or wheat bran and combined with all of them

Reduction % of green and blue molds on orange fruits in response to protecting effect of *S. cerevisiae* grown on wheat bran or soybean alone or in combination during storage at 20°C after 20 days.Data in Table (4) indicated that all treatments of *S.cerevisiae* , wheat bran and soybean alone or in combination were effective on protective orange fruits . Application of *S.cerevisiae* with wheat bran : soybean rate (4:6) or soybean alone on each green mold causes 100% reduction in D.I. and D.S. after 20 days compared with *S.cerevisiae* , wheat bran , individually were 70.7,73.3,75.0,and 86.7) respectively.

The same trend on blue mold the most effective treatment was soybean alone or *Saccharomyces cerevisiae* with wheat bran +soybean rate 4:6 completely reduced D.I. and D.S.100% reduction. But on *S.cerevisiae* , wheat bran , individually were(80.8,87.2,86.7 and90.0)respectively.

In vivo :Effect of Lime fruits coating with *S. cerveciae* formulated on soy bean or wheat bran and combined with all of them

Data in Table (5) showed that the same trend of results followed by the same treatment. All treatments reduced disease severity and

disease incidence in comparison with control. Promising treatment with *S.cerevisiae* with wheat bran +soybean causes the highest reduction in green and blue molds on lime fruits (86.2, 93.3, 84.0, and 95.0) respectively .

The potential of using wheat bran or soybean supplemented with *S.cerevisiae* to control green and blue molds on citrus fruits resulted in significant reduction. This combination had a suppressive effect on decay incidence of citrus fruits resulted in significant reduction compared with the other treatment applied individually. Many workers also successfully used different yeast isolates for controlling post harvest diseases during storage. Scientists reported that treatment of fruits with yeast was an efficient method for control of several post-harvest decays[8, 31,32, 33, 34].

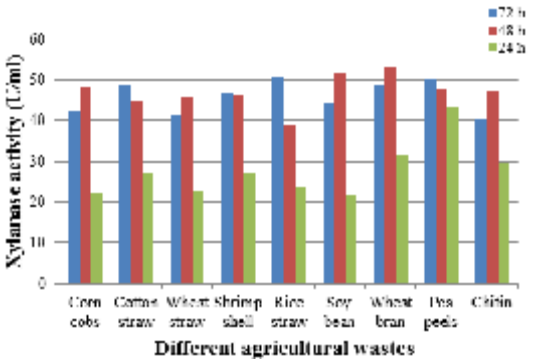

Fig. 1. Effect of different incubation periods (hours) on xylanase production by *S. cerevisiae* using different agricultural wastes

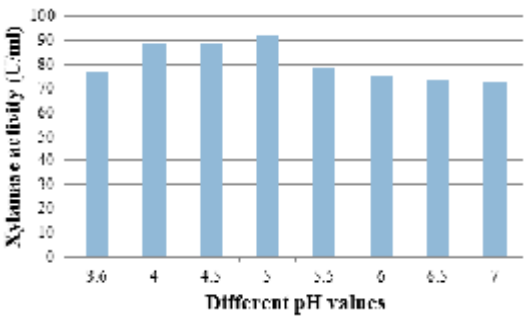

Fig. 2. Effect of different pH values on xylanase production by *S. cerevisiae*

SDS-PAGE gel electrophoreses of crude xylanase enzymes

SDS-PAGE of the crude xylanase from *S. cerevisiae* produce band of molecular weight 74 KDa. This results were inagreament with[35] who found that crude xylanase obtained from *Streptomyces sp.* (strain Ib 24D)was 58KDa .The molecular weight of endoxylanases from several *Streptomyces sp*.was in the range 25-50 kDa this was reported by[36]. Molecular weight of endoxylanase from *S.thermo violaceus* was 54 kDa[37].

CONCLUSION

The present study demonstrates that *Saccharomyces cerevisiae* grown on wheat bran + soy bean (4:6) was most effective for xylanase production under optimum condition and have potential as environmentally friendly nontoxic post-harvest fungicides for citrus molds control and could be suggested for commercial use in packing houses in consideration to their wide consumption as safety food.

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