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Full Length Research Paper

Valorization of Pomegranate Peel for Invertase Production by a Marine Strain of *Aspergillus terreus*

Aliaa R. El-Shamy and Sherien M.M. Atalla

Department of Chemistry of Natural and Microbial Products, National Research Centre, Giza, Egypt

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Agricultural wastes were the low cost raw material for invertase production by fungi. The marine fungal strain *Aspergillusterreus* was characterized and phylogenetic analysis by 18S rRNA gene sequencing then evaluated for invertase production using 40 g/l of pomegranate peel (Pp) as substrate. Maximum invertase activity was 264.4 U/ml after 5 days incubation period. Two disks 6mm in diameter, pH 6.5 and 30 °C were the optimum conditions for invertase production. 3% of triton x100 as surfactant produce 281.2 U/ml .Increasing invertase production by addition of wheat bran ,EDTA and cysteine to pomegranate peel.

Keywords: marine fungi, invertase, pomegranate peel, optimization.

INTRODUCTION

Enzymes are sort of protein in all living things which act as biological catalysts, increase the rate of chemical reactions without undergoing any permanent change themselves (Tamio et al., 2009).

Invertase is a special kind of enzyme, which catalysisα-1,4glycosidic linkage between α-D-glucose and β-D-fructose molecules of sucrose by hydrolysis releasing monosaccharide's such as glucose and fructose. This mixture is called invert syrup. It also hydrolyses β-fructanse such as raffinose into simple sugars (Ashokkumar and Gunasekaran 2002). In addition, they exhibit transfructosyl activity at high sucrose concentrations (Kotwal and Shankar 2009; Guimaraes et al., 2007).

Invertase is one of the most widely used enzymes in food industry where fructose is preferred in the preparation of jams and candies because it is sweeter and does not crystallize easily, thus making these enzymes suitable for biotechnological applications (Uma et al., 2010).Invertase is extensively used in confectionery, food industries and in pharmaceuticals (Ashokkumar et al., 2001).It has wide range of commercial applications including the production of confectionery with liquid or soft centers, chocolates and fermentation of cane molasses into ethanol. Invertase used in pharmaceutical industry as digestive aid tablets, powder milk for infants foods, as calf feed preparation, assimilation of alcohol in fortified wines

*Corresponding Author's Email: Aliaa.ragab@hotmail.com; Atallam@mailcity.com

and in manufactured inverted sugars as food for honeybees (Rashad and Nooman 2008).

The microbial sources are preferred over other sources of invertase to meet commercial needs (Abdulsattar et al., 2012).

Many microorganisms produce invertase and can utilize sucrose as a nutrient, such as *Neurosporacrassa, Candida utilis, Fusarium oxyspoum Phytophthorame ganosperma, Aspergillus niger, Saccharomyces cerevisiae, Schizosaccharomycespombe and Schwanniomyces occidentalis* (Silveira et al., 2000).The microbial sources are preferred over other sources of invertase to meet commercial needs(Abdul sattar et al., 2012).

The aim of this work was production of invertase from marine fungalstrain *Aspergillus terreurs* using pomegranate peel under optimum conditions.

MATERIALS AND METHODS

Microrganism and inoculum preparation

Marine fungal strain *Aspergillus terreus* was isolated from red sea water at Ain Sokhna and was screened for invertase production from 7 days old culture.

Identification of microorganism:

Isolation of genomic DNA and 18S RNA sequencing DNA extraction was done by using protocol of Gene Jet genomic DNA purification Kit (Thermo K0721) as following:

Genomic DNA was harvested up to 2x10^9 fungal spores in a 1.5 or 2 ml micro centrifuge tube by centrifugation for 10 min at 5000 x g. This discard the supernatant was resuspend the pellet in 180µl of digestion solution then 20µl of proteinase k solution and mixed thoroughly by vortex or pipetting to obtain a uniform suspension. Sample was incubated at 56°C while vortex occasionally until the cells are completely lysed (~30 min).

Add 20µl of RNase to the solution, mix by vortex then incubate the mixture for 10 min at room temperature. Then 200µl of lysis solution was added to the sample mixed thoroughly by vortex for about 15 second until a homogeneous mixture was obtained. Add 400µl of 50% ethanol was added and mixed then transferred the prepared lysate to a GeneJET™ Genomic DNA Purification Column inserted in a collection tube, centrifuge the column for 1 min at 6000 x g. Discard the collection tube containing the flow through solution.

Place the GeneJET™ Genomic DNA Purification Column into a new 2 ml collection tube (included),then add 500µl of wash buffer I. Centrifuge for 1 min at 8000 x g. Discard the flow through and place the purification column back into the collection tube. Add 500µl of wash buffer II to the GeneJET™ Genomic DNA Purification Column. Centrifuge for 3 min at maximum speed (≥12000 x g). Discard the collection tube containing the flow through solution and transfer the GeneJET™ Genomic DNA Purification Column to a sterile 1.5 ml micro centrifuge tube. Then add 80µl of elution buffer to the center of the GeneJET™ Genomic DNA Purification Column membrane to elute genomic DNA. Incubate for 2 min at room temperature and centrifuge for 1 min at 8000 x g.

PCR amplification of 18S *rRNA* gene:

PCR amplification of 18S rRNA gene from purified DNA was done by using Maxima Hot start PCR Master Mix (Thermo) K0211using Primer set:

ITS1 TCC GTA GGT GAA CCT GCG G
ITS4 TCC TCC GCT TAT TGA TAT GC

The reaction condition was as follows:

Initial denaturation 95°C for 10 minute then 95°C for 30 second, Annealing 55°C for 1 minute and primer extension 72°C for 1minute followed by a final extension 72°C for 15minute.
No. of cycles =35

PCR product purification:

The unpurified DNA was purified using PCR clean up to PCR product using Gene JET PCR Purification Kit (Thermo K0701) by adding 45µl of binding buffer to complete PCR mixture. The mix was transferred from step one to the Gene JET Purification Column, centrifuged for 30-60 second at 12000xg then add 100µl of wash buffer to the Gene JET Purification Column for 1 min. Transfer the Gene JET Purification Column to a clean 1.5 ml micro centrifuge tube then add 25µl of elution buffer to the center of the Gene JET purification column membrane for 1 min. Discard the Gene JET Purification Column and store the purified DNA at -20°C.

Sequencing:

Sequencing to the PCR product on GATC company by use ABI 3730xl DNA sequenced by using forward and reverse primers.

Phylogenetic analysis:

A phylogenetic analysis of the isolate was performed to determine how the 18S rRNA sequence of the isolate and related strain might have been derived during evolution. The evolutionary relationships among the sequences were depicted by placing them as outer branches on a phylogenetic tree. The branching relationships on the inner part of the tree reflect the degree to which different sequences are related. Combining the traditional sanger technology with the new 454 technology, can genomes now be sequenced and analyzed in half the usual project time, with a considerable reduction in the number of coatings and gaps. The analysis of nucleotide sequence was done in Blast alignment (www.thermoscientificbio.com).

Fermentation conditions:

The medium used for invertase production according to (Uma et al., 2012), consist of (g/l): 500ml of sterile distilled water and 500 ml of sea water was mixed with yeast 10.0, ammonium sulphate 1.0, magnesium sulphate 0.75, potassium dihydrogen phosphate 3.5, sucrose 20 which replaced by pomegranate peel, pH 5.5 . Erlenmeyer flask containing 50 ml fermentation media was inoculated with 2 disks (6mm) in diameter and incubated for 7 days at 28 ◦C under static condition.

Enzyme assay:

Invertase activity was determined using the method of (Aranda et al., 2006), was modified by incubating 0.1 ml buffer 0.9 ml of sucrose in 0.03 M acetate acid pH 5.0,incubated at 50◦C for 30 min. To stop the reaction 1 ml of dinitrosalicylic acid was added then boiling water bath for 5 min. The absorbance was read at 540 nm.

Optimization of fermentation media:

Various process parameters influencing enzyme production including different agricultural wastes, different incubation periods (2-12) days, different pH values (3.5-8.0), different inoculums size, different temperatures, surfactant, different additives and different metal ions were studied.

RESULTS AND DISCUSSION

Morphological identification of the fungal isolate:

The marine fungal strain *Aspergillusterreus* was purified by repeated sub-culturing on the potato dextrose agar medium at regular intervals and incubating at 29◦C. The isolate was identified based on the colony morphology, microscopic observation and molecular identification (Collier et al., 1998).

Identification and phylogenetic position of fungal isolate:

The identification was done based on 18S rRNA gene sequencing. The 18S rRNA sequences of the isolate was compared with the data present in NCBI. The BLASTn of the isolate was showing 100% homology with *Aspergillus* sp. Figure (1) showed the phylogenetic relationship between the isolate and other related fungi. The homology assay result indicated the isolate was in the phylogenetic branch of *Aspergillus*sp.

Effect of different agricultural wastes on invertase production:

Different agricultural wastes were used as carbon source (olive mill waste, soy bean, pomegranate peel wastes, pisumsativum waste and wheat bran) invertase production were studied in comparison with sucrose as a control. Results in Table (1) show that pomegranate peel (Pp) give the highest invertase activity 183.0 U/ml followed by sucrose 131.2 U/ml then by olive mill waste which give 119.5 U/ml .These results were the same with (Uma et al., 2012), who proved that pomegranate peel was found to be the best substrate for production of invertase using *Cladosporiumcladosporidies* (Uma et al., 2010).

Different concentrations of pomegranate peel (Pp):

The effect of different concentrations of pomegranate peel Pp (5 to 50g/l) on invertase production from the marine isolated fungal strain *A.terreus* were studied. Results in Figure (2) showed that increasing in Pp concentrations result in increasing invertase activity, the maximum activity was at concentration 40 g/l giving 264.4 U/ml .Pineapple peel (Pp) can be more effectively as a substrate for invertase production when added in a concentration 20g/l using *Aspergillusniger* and *Aspergillusflavus* (Revathi and Uma 2014).

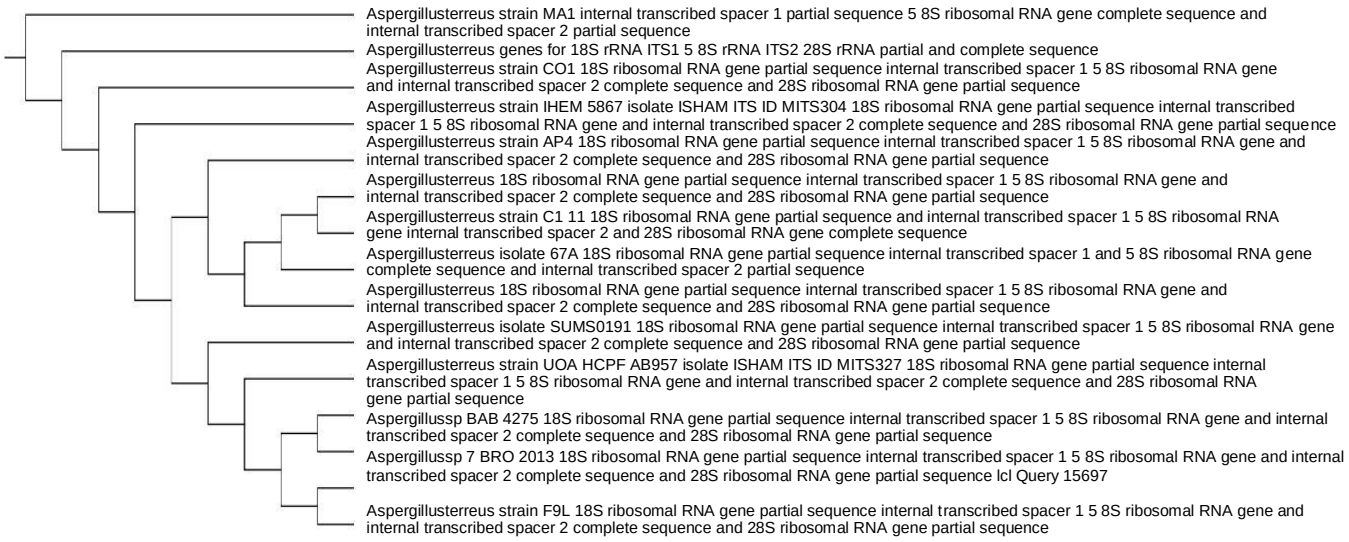

Figure 1:Phylogenetic tree of *A.terreus* based on 18S ribosomal RNA gene sequencing showing homology with *Aspergillus sp.*

Table 1: Effect of different agricultural wastes on invertase production by marine fungal strain*A. terreus*.

Different agricultural wastes	Activity (U/ml)
Sucrose	131.2
Olive mill waste	119.5
Soy bean	65.5
Pomegranate peel (Pp)	183.0
Pisumsativum waste	91.6
Wheat bran	63.2

Different incubation periods:

The effect of incubation periods on invertase production were carried out by marine fungal strain *Aspergillusterreus* from (2:12) days under static condition. Results in Figure (3) showed that activity increase with increasing incubation time till reached 5^{th} day 264.4 U/ml followed by 7^{th} day produce 253.4 U/ml. Then activity of enzyme decrease with increasing time of incubation. This results was in agreement with (Uma et al., 2012),who found that *C. cladosporioides* produce maximum invertase activity after 4 days of incubation. Invertase by *Aspergillus flavus* was reached its maximum activity between the 72-92 hours of time rests(Uma et al., 2010). Time course study was one of the most critical factors, which governs the value of the process along with product formation (Laluce 1991).The pattern of accumulated reducing sugar after specific incubation time is characteristic to the each species, the growth and enzyme yield increased with passage of time and the maximum enzyme secretion was noted (35.89 U/ml) after 48 h. but on prolong incubation production decreased as discussed by (Abdulsattar et al., 2012).

Different inoculum size:

To evaluate the effect of different inoculum size on invertase production by marine fungal strain *A.terreus*. Fermentation media was inoculated with 2 disks from (4.0-12.0) mm in diameter and incubated for 5days at 28-30 ˚C. Results in Figure (4) showed that inoculation

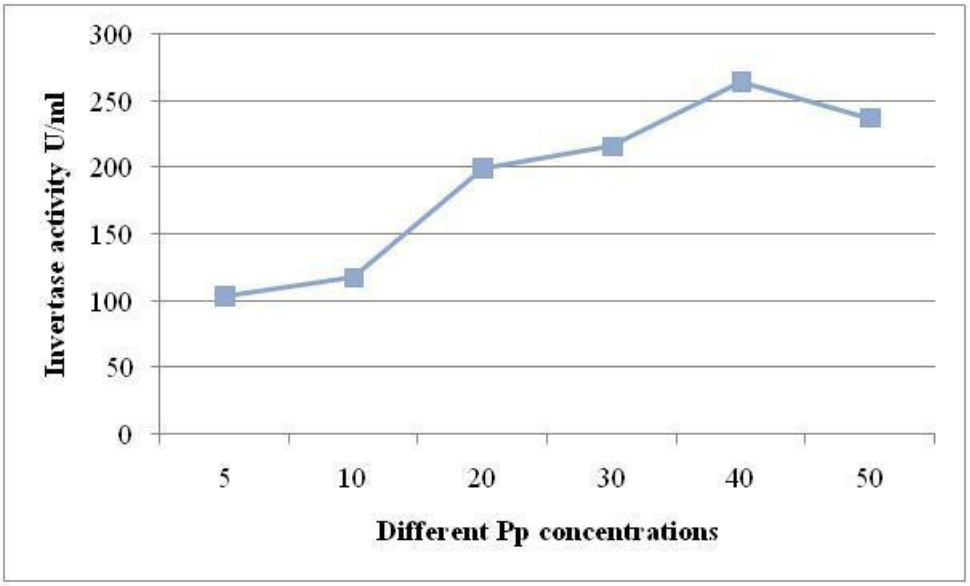

Figure 2: Effect of different concentrations of pomegranate peel (Pp) on invertase production by *A.terreus*.

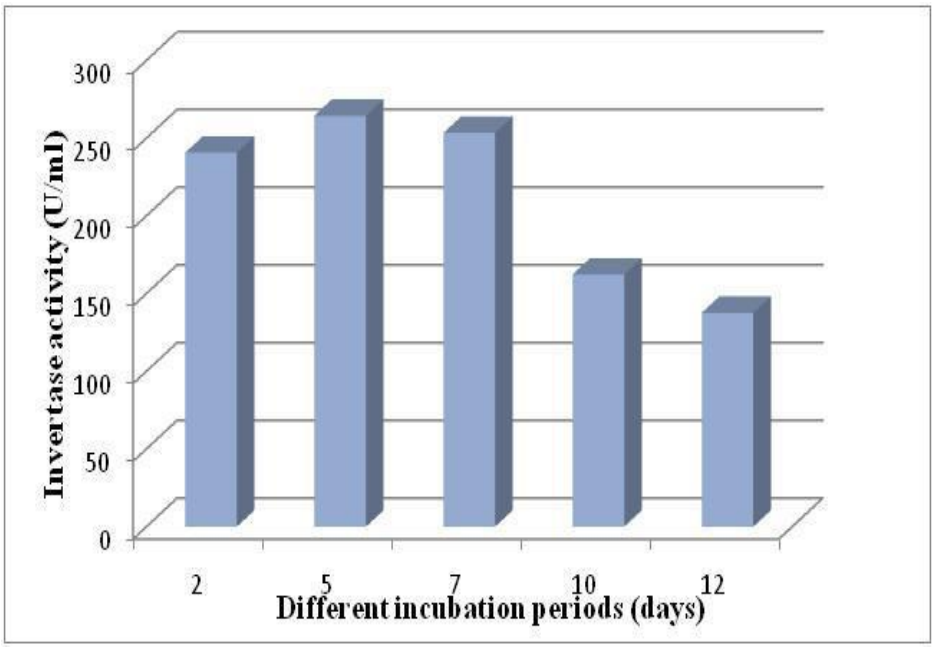

Figure 3: Effect of different incubation periods on invertase production by *A.terreus*.

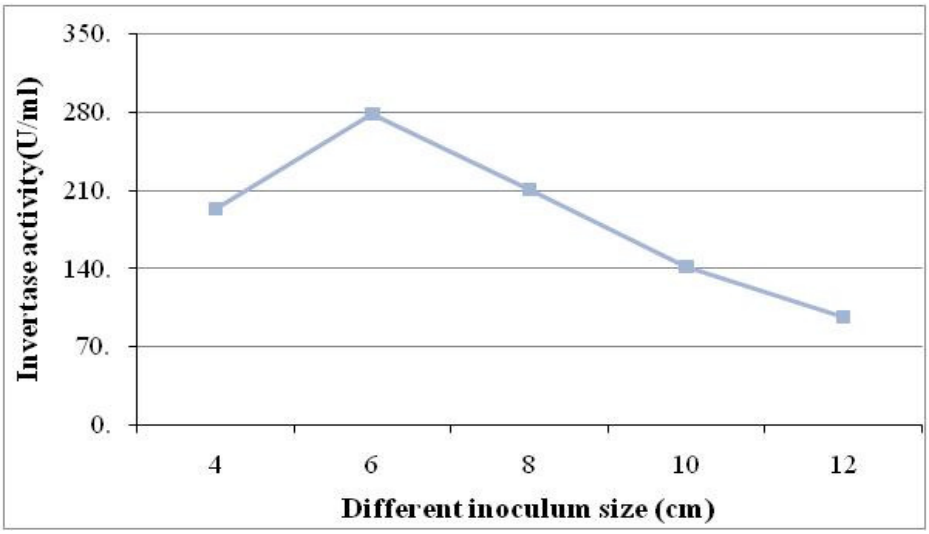

Figure 4: Effect of different inocculum size on invertase production by *A. terreus.*

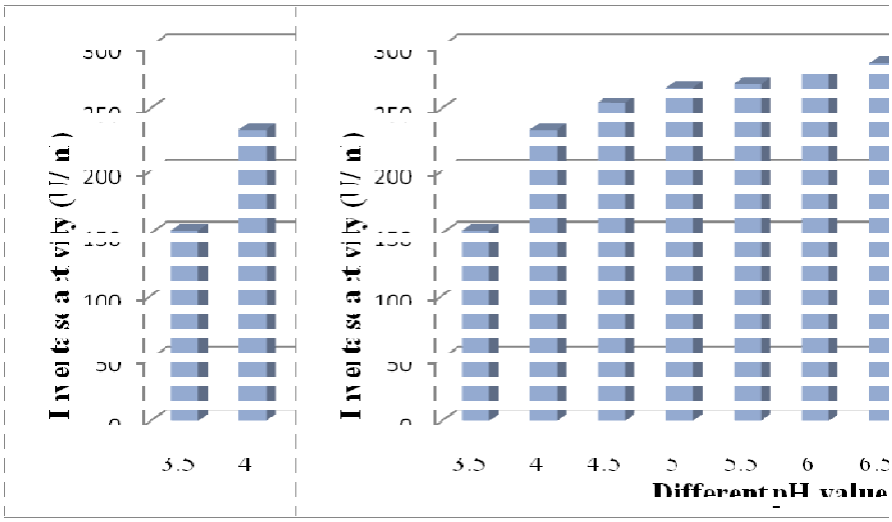

Figure 5: Effect of different p H values on invertase production *by* A. *terreus*.

with disks 6 mm in diameter produce maximum invertase activity 277.9 U/ml, with increasing inoculum size enzyme activity begin to decrease**.** The inoculum size was further increased, the productioon of the enzyme gradually decreased due to the faact that at high level of inoculum size fungi grow fast by consuming the essential nutrients at the inittial stages and rapid accumulation of by product into the fermentation medium also (Ul-Haq and Ali 2005), observed that the reason for the low prodduction of enzyme at the inoculums size below optimumm was due to the slow growth of the organism and extended time period to utilize nutrients properly. Inoculum size larger than optimum of *Aspergillus flavus* caused overgrowth, nutrient imbalance resulting in less enzyme production (Uma et al., 2010).

Different pH values:

Different pH values from (3.5-8.0) afffected on invertase production , invertase activity increase with increasing pH values till reached its maximum activity at pH 6.5 produce 277.9 U/ml then activity beggin to decrease as shown in Figure (5). This results wa s coincided with

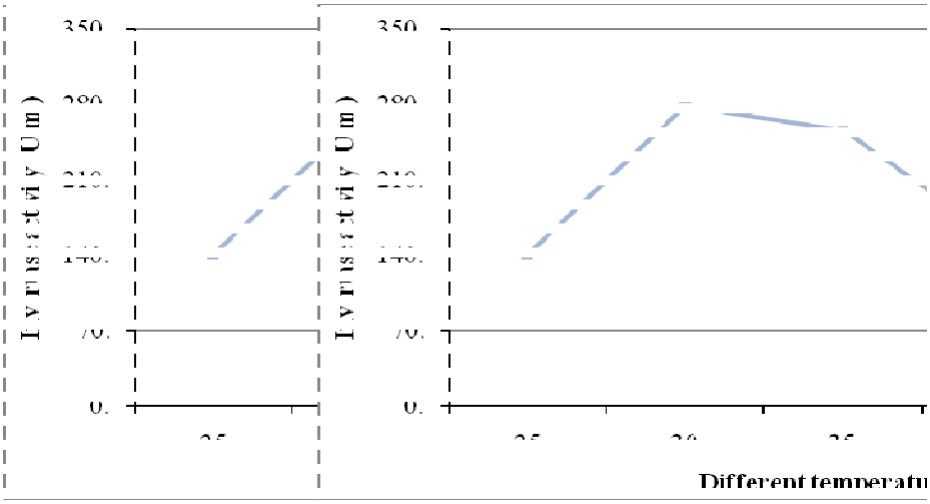

Figure 6: Effect of different tem perature on invertase production *by A. terreus.*

Table 2: Effect of different surfactant on invertase production by *A.terreus*.

Different surfactant	Activity (U/ml)
Triton x100 (11%)	267.1
Triton x100 (22%)	276.2
Triton x100 (33%)	281.2
Tween 80 (1%)	175.0
Tween 80 (2%)	201.4
Tween 80 (3%)	184.9

(Abdulsattar et al., 2012), who found that the maximum pH values from *Mucorgeophillus* EFRL 03 was at pH 6.5. found that the maximum invertase activity from was at pH 4.0 (Uma et al., 2010).Maximum invertase activity from *C. cladosporioides* and *A. flav us* was at pH 4.0 and 5.0 respectively as discussed b y (Uma et al., 2010).The higher enzymatic activity from *Aspergillusnidulans* and *Emericelanidulans* were at pH of 4.8 - 5.6 respectively, less enzyme production at developed pH was due to blocked enzyme secretion from the yeast cells(Juliana et al., 2013).Thee optimum pH from the marine *psychrophilic* and endemic Antarctic *Leucosporidiumantarcticum* strain 171 was 4.55 to 4.75 as shown by (Marianna et al., 2005).

Different temperatures:

Temperature play an important role in pro duction of invertase enzyme. Among the various temperature from (25-45◦C), 30◦C was the optiimum temperature as shown in Figure (6). Increasing the degree of temperature resulted in inhibition of the activity of the enzyme. This results were in acccordance with the results reported by (Uma et al., 20100), who proved that invertase release from *Aspergillusflaavus* was activated at 30◦C. At high temperature the ennzyme activity was not significant because of rising the temperature resulting in denaturation of enzyme active site (Russo et al., 1996).

Different surfactant:

The effect of two different kinds of surfactant at different concentrations on invertasse production was tested after 5 days of incubation period at 30˚C. Results in Table(2) proved that the highest invertase activity was noticed when the prroduction medium contained triton x 100 at concentration 3% followed by

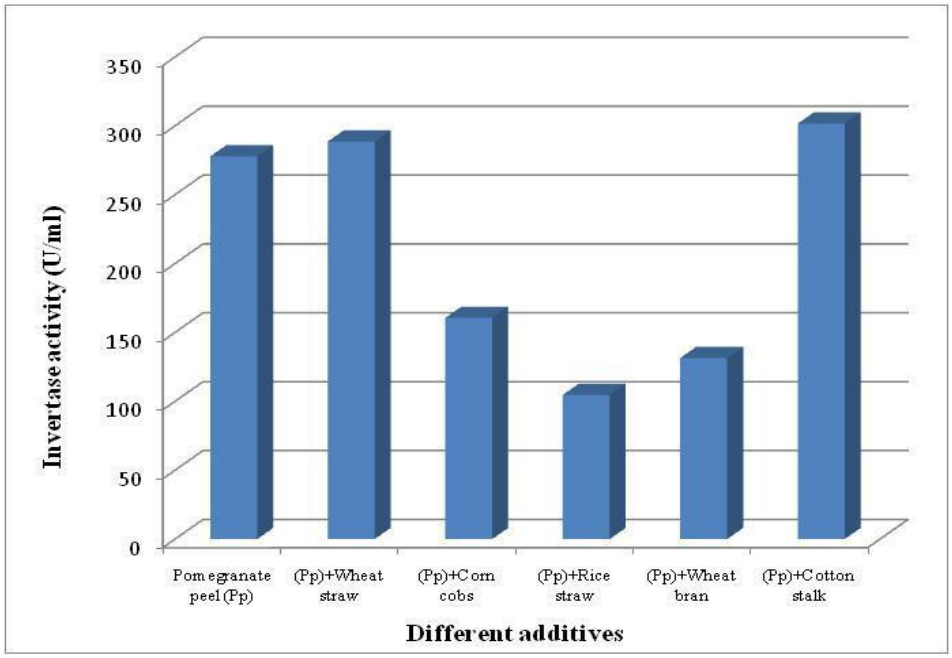

Figure 7: Effect of different additives on invertase production by *A. terreus.*

Triton x 100 at concentration 2%, the smallest invertase activity was given when the culture medium contain tween 80 at concentration 1%. Invertase production from *Penicilliumchrysogenum,* has no effect of enzyme production was observed upon triton x 100,slightely increase in enzyme activity observed between 2,4,6,8 %.Surfactant may affect the growth rate of enzyme production of many fungi by enhancing extracellular enzyme production lead to increase cell permeability, change in lipid metabolism and stimulating the release of enzyme as reported by (Fadel and El Batal 2000).

Different additives on invertase production:

The aim of this experiment was increasing invertase production by adding different agricultural wastes (wheat straw , corn cobs ,rice straw ,wheat bran and cotton stalk) to pomegranate peel (Pp). As shown in Figure (7) Pp with cotton stalk produce maximum invertase activity 301.6U/ml followed by Pp with wheat straw 288.6 U/ml while Pp with rice straw produce minimum activity. Agro industrial wastes have been used as carbon, hydrogen and oxygen sources to produce ethanol, proteins and microbial enzymes stated by (Shankar et al., 2013).Filamentous fungus Rhizopussp. cultivated on wheat bran medium actas polyacrylamide geland produce maximum invertase activity as identified by (Goulart et al., 2003).

Effect of different metal ions on invertase production by *A.terrus:*

Different metal ions was tested for their ability to activate or inhibit the activity of invertase using Pp waste by *A.terreus* .Results in Table (3) showed that cystine and EDTA was most promising for enzyme activation produce 295.1and 298.6 U/ml respectively on the other hand $ZnSO_4$ and $CuSO_4$ produce the minimum activity, other metal ions produce moderate to low activity. This results was coincided with (Uma et al., 2012), who found that Zn^{2+} and Cu^{2+} inhibit enzyme activity of the fungal strain *Aspergillusflavus,* this means that invertase activity inhibited while incubating with highly heavy metal ions.

Table (3): Effect of different metal ions of pomegranate peel on invertase production by *A.terreus*.

Different metal ions	Activity (U/ml)
Control	277.9
EDTA	298.6
$CuSO_4$	58.8
$ZnSO_4$	54.0
$MgCl_2$	116.2
$CoCl_2$	136.0
$HgCl_2$	115.3
$CuCl_2$	37.1
Cystine	295.1
L-cystiene	284.6

CONCLUSION

Production of invertase from marine fungal strain *A. terreus* using 40 g/l pomegranate peel as substrate at 6.5 pH, 30 ˚C and 3 % triton x 100 . Addition of cotton stalk, cystine and EDTA were most promising for increasing invertase activity.

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