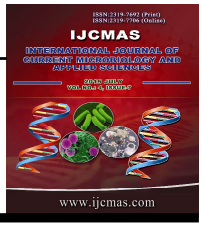
Int.J.Curr.Microbiol.App.Sci (2015) 4(7): 511-524

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 7 (2015) pp. 511-524**
http://www.ijcmas.com



Original Research Article

Production of Bioethanol Via Microbial and Enzymatic Hydrolysis of Potato Wastes Under Solid State Fermentation

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A B S T R A C T

A potato wastes powder utilizing bacteria was isolated from soil. Based on morphological, physiological and 16S rDNA, this bacterium was identified as *Bacillus subtilis*. Inoculation of the solid state culture on potato wastes powder at pH 6.5 with 20% inoculum size of *B. subtilis* at 30ºC resulted in the maximum biomass and amylase activity. The maximum biomass of *B. subtilis* and its α-amylase production was achieved in the stationary phase; α-amylase production coincided with the increase in the specific growth rates of the cultures and reduction of starch content in potato wastes powder. *B. subtilis* was able to degrade starch content in solid potato wastes completely in solid state culture at pH 6.5 and 30 °C after 4 days. Potato wastes powder exhibited different susceptibilities towards amylase to their conversion to reducing sugars. The present study showed also that, the general trend of potato wastes powder bioconversion with *B. subtilis* amylase was more than the general trend by *B. subtilis*. This enzyme effectively led to enzymatic conversion of *B. subtilis* pretreated starch from potato wastes powder into glucose, followed by fermentation into ethanol. Maximum ethanol production from the hydrolysate of potato wastes powder was at 30 °C, pH 4.5 and inoculum size 10 % after 3 days of incubation. The utilization of amylase and *Saccharomyces cerevisiae* for ethanol production from the potato wastes under the optimum conditions can not only utilized for cheap clean energy production, but also for getting rid of some undesired wastes.

Keywords

Potato wastes, *Bacillus subtilis*, Amylase, Hydrolysis, Solid state fermentation

Introduction

Due to gradual decrease of fossil fuels, bioethanol has got the attention of many investigators to use it as alternative source of energy across the world. Bioethanol is produced from the fermentation of renewable resources for fuel and considered to be produced from biomass based materials. Most of the countries have either ethanol blended gasoline or direct ethanol as fuel (Grassi, 1999 and Sehsah *et al.*, 2014).

Lignocellulosic biomass is option for ethanol fermentation but this substrate is complex and requires expensive pre-treatments. Currently, potatoes are an alternative feedstock for ethanol production.

Potatoes are the second most used food in the world. Potatoes are starchy crops which do not require complex pre-treatments. Moreover, during processing of potato, particularly in the potato chip industry, approximately 18% of the potatoes are generated as waste. Different of potato wastes potato industry can be a good carbon source for yeast during alcohol fermentation (Fadel, 2000; Yamada *et al.,* 2009 and Adarsha *et al.,* 2010). Amylases (α-amylase, β-amylase and glucoamylase) are employed for hydrolysis of starchy materials. α-Amylase hydrolyses the 1,4-α-D-glucosidic linkages in the linear amylase chain, randomly. However, amyloglucosidase cleaves the 1,6-α-linkages at the branching points of amylopectin as well as 1,4-α-linkages. Amylases are of great significance in biotechnological applications ranging from food, fermentation, detergent, pharmaceutical, brewing and textile to paper industries (Pandey *et al.,* 2000). Production of these α amylases has been investigated through submerged (SmF) and solid-state fermentation (SSF). However, the contents of a synthetic medium are very expensive and uneconomical, so they need to be replaced with more economically available agricultural and industrial by products, as they are considered to be good substrates for SSF to produce enzymes (Sodhi *et al.,* 2005; Rameshkumar and Sivasudha, 2011; Saxena and Singh, 2011). The solid-state fermentation (SSF) process has been developed and used more extensively. It has advantages over SmF like simple technique, low capital investment, cheaper production of enzyme having better physiochemical properties, lower levels of catabolite repression and better product recovery (Baysal *et al.,* 2008; Belal, 2003; Belal and El-Mahrouk, 2010; Belal and Khalafalla, 2011; Belal, 2013a). *Bacillus* strains such as *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* are known as good producers of alpha-amylase for various applications (Belal *et al.,* 2014). Potato in Egypt is one of the most important crops grown for local consumption, export and processing. Potatoes production 2013 was 4.8 million tons from 148,000 Hectare with an average of 26.9663 tons per Hectare (FAO, 2014). Different methods created for potato wastes utilization were reported by many investigators (Khan *et al.,* 2012; Duhan *et al.,* 2013; Sehsah *et al.,* 2014; Belal *et al.,* 2014). So, the aim of the present study is designed to isolate and characterize of the potato wastes degrading microorganisms and production of bioethanol via microbial and enzymatic hydrolysis of potato wastes under solid state fermentation.

Materials and Methods

Isolation and selection of the highest amylase-producing strain

Samples of soil were collected from different localities in Kafr El-sheikh Governorate, Egypt. Samples of soil were added as 10 g to 90 ml of sterilized distilled water. The flasks were subjected to shaking for 30 min on a rotary shaker (150 rpm) at room temperature. Dilution series were prepared in a glass test tube containing 9 ml distilled water up to $1:10^{-6}$. Ten-fold dilutions were prepared and then 100 µl was transferred onto nutrient agar (Beef extract (3 g/l), peptone (5 g/l), starch (10 g/l), Agar (15-20 g/l)) plates containing starch (10 g/L) and spread evenly with glass triangle. The plates were sealed in polyethylene bags and were incubated at 30 ˚C for 7 days monitored for appearance of colonies. After 72 h incubation at 30 ºC, isolated colonies were transferred to fresh nutrient agar plates containing starch and allowed to incubate for another 72 h at 30ºC. Plates were then

flooded with 1 % Gram's iodine solution and the isolate producing wider clear zone of hydrolysis was retained for further studies. Pure culture was obtained and maintained through repeated streaking of the isolated strain on fresh nutrient agar medium containing starch. The pure culture stocks of these isolates were stored at 4 °C on nutrient agar medium slopes containing starch (10 g/L). The bacterial isolate which formed the widest clear zone was selected as the most efficient isolate in producing amylase. At least three independent experiments were performed with the unspotted plate as a control. All experiments were performed in triplicates.

Identification

The efficient selected starch degrading strain (designated as E19) was identified depending morphological and physiological characteristics as described by Parry *et al.*, (1983) and 16S rDNA sequencing as follow:

The amplified product of approximately 1254 bp (1,254 bp for nested PCR) was carried out according to Claudio *et al.*, (2002). Sequencing was performed using a Big Dye terminator cycle sequencing kit (Applied BioSystems, Foster City, CA). Sequencing products were purified by using Centri-Sep™ Columns (Princeton Separations, Adelphia, NJ) and were resolved on an Applied BioSystems model 3100 automated DNA sequencing system (Applied BioSystems).

The evolutionary history was inferred using the Minimum Evolution method according to Rzhetsky and Nei (1992). The optimal tree with the sum of branch length = 0.67162741 was shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method according to Tamura *et al.* (2004), and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) and a search level of the Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree. The analysis involved 43 nucleotide sequences. Codon positions included were 1st+2nd+ 3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 765 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2013).

Amylase production in solid state fermentation

Preparation of substrate

Potato wastes and rotted potatoes were collected from the market or household wastes and refrigerators keeping fruit and vegetables of Kafr-El-Sheikh and El-Gharbia Governorates, Egypt. The samples were placed after removing the rotted parts in polyethylene bags, sealed, transferred to the laboratory. These waste materials were peeled and cut into pieces, washed, spread on trays and oven dried at 70°C. The dried wastes were ground in mixer grinder and stored in polythene bags at room temperature.

Solid state fermentation method was used since it consumes lesser power but produce more concentrated product. Fifty grams of potato wastes powder were inserted into each 500 mL Erlenmeyer flasks with cotton stopper. Each flask was finally inoculated with 10 ml of a cell suspension of *B. subtilis* (nutrient broth medium, 10^7 cfu/ml,

incubated at 30°C and 150 rpm for 3 days). Potato wastes powder was moistened to 65% moisture content with nutrient broth medium containing mineral salts solution ($MgSO_47H_2O$, 0.1%; KH_2PO_4, 0.1 %; $CaCl_2$, 0.1 %; $FeSO_4$, 0.05 % and $(NH_4)_2SO_4$, 0.1 %). After mixing, flasks were incubated at 30 °C under static conditions for 6 days (Belal, 2013a). The contents of the flasks were mixed and autoclaved at 121°C for 20 min and mixed manually daily during incubation time.

Determination of optimal pH and temperature for *B. subtilis* amylase production was investigated in solid state fermentation. The optimum pH for amylase production was estimated at various pH values 6, 6.5, 7, 7.5 and 8 and with appropriate buffer at 30 °C for 6 days. For determination of optimum temperature for amylase production, the reactions were carried out at 20, 30 and 40 °C and pH 6.5 for 6 days.

To investigate the effect of the inoculum size on *B. subtilis* growth and amylase production, SSF medium of potato waste powder was inoculated with different amounts of bacterial inocula [5, 10, 15 and 20 % of bacterial culture (10^7 cfu/ml)] were added to the prepared flasks. The experiments were incubated at pH6.5 and 30 °C for 6 days.

Growth medium containing 50 g of potato waste powder was inoculated with different amounts of bacterial inocula [20 % of bacterial culture (8 x 10^7 cfu/ml)] and incubated for varying time periods (1, 2, 3, 4, 5, and 6 day) at pH 7 and 30°C. Starch content was estimated daily by the method described by Aullybux and Puchooa (2013).

Amylase extraction was done by adding 100 ml of 0.1 M acetate buffer (pH 6.0) into each flask and the mixture incubated at 30°C on an orbital shaker, at 200 rpm for 1 h. The suspended slurry was centrifuged at 10000 rpm for 20 min at 4°C and filtered using sterile membrane filter (0.2 mm). The supernatant was used as the crude enzyme solution (Belal, 2003; Belal and El-Mahrouk 2010; Belal, 2013a). Amylase activity was determined as described below. Numbers of bacterial cells in each culture were determined by plating appropriate dilutions onto nutrient agar medium. The protein concentration of enzyme was determined according to Lowry *et al.* (1951). All experiments were performed in triplicates. Control flasks of equal volume of media and starchy waste.

Enzyme assay

Alpha amylase activity was estimated using DNS (3, 5 dinitrosalicylic acid) method. Slurry of concentrations of potato waste powder (30 % w/v) was prepared in water and autoclaved. Assay of **α**--amylase activity. **α**-amylase activity was determined by incubating 1 ml of an appropriately diluted enzyme solution with 5 ml of 30% autoclaved dried potato waste slurry solution, 1 ml of deionized water and 1 ml of 0.1 M acetate buffer (pH 6.0) at 60°C for 1h with agitation. The reaction was stopped by adding DNS and kept in boiling water bath for 10 min. The absorbance was read at 540 nm using a spectrophotometer against glucose as the standard. In these tests, reducing sugars were estimated calorimetrically after Miller (1959), using glucose as standards. The crude enzyme concentration was determined according to Lowry *et al.* (1951). One unit of enzyme activity is defined as the amount of enzyme, which releases 1µmole of reducing sugar as glucose per minute, under the assay conditions (U/ml/min). The experiments were carried out in triplicates.

Bioconversion of potato waste powder into bioethanol using *B. subtilis* amylase

Microorganism

B. subtilis as efficient potato waste powder degrading microorganism was used for solid state fermentation as described above.

Enzymatic hydrolysis

The ability of the crude enzyme to hydrolyze raw starch was studied using potato waste powder. Slurry of concentrations of potato waste was prepared as described above. Crude *B. subtilis* amylase solution, (1 ml); 1 ml of 0.1 M acetate buffer (pH 6.0) and 5 ml of 30 % autoclaved dried potato waste slurry solution were incubated at 60^{o}C for 1 h with agitation. Susceptibility of the potato waste powder to hydrolysis was determined in terms of the quantity of reducing sugars produced. The reaction was monitored by the yield of total reducing sugars estimated by dinitrosalicylic acid method (Miller, 1959).

Optimization of fermentation conditions

Effect of inoculum size

To investigate the effect of the inoculum size, the hydrolysate (after hydrolysis with *B. subtilis* amylase) was inoculated with different concentrations of inoculum that is 5, 10, 15 and 20% (v/v) of *S. cerevisae* culture with colony count at 10^8 cfu/ml and kept for fermentation at pH4.5 with shaking at 200rpm 30°C for 4 days.

Effect of incubation period on bioethanol production

For studying the effect of the incubation period, the hydrolysate (after hydrolysis with *B. subtilis* amylase) was inoculated with 10% of *S. cerevisae* culture with colony count at 10^8 cfu/ml as described below. The fermentation was carried out at pH4.5, with shaking at 200 rpm and 30°C for 4 days. Ethanol content in fermented sample was estimated daily as described below.

Effect of pH and temperature on bioethanol production

The pH of hydrolysate (after hydrolysis with *B. subtilis* amylase) was adjusted to different levels (4, 4.5, 5, 5.5, 6, 6.5 and 7) and it was fermented after inoculation with 10% of *S. cerevisae* culture with colony count at 10^8 cfu/ml as described below. The fermentation was carried out at 30°C and shaking at 200rpm for 3 days.

Effect of temperature on bioethanol production

To determine the optimum temperature, the hydrolysate (after hydrolysis with *B. subtilis* amylase) was inoculated with 10% of *S. cerevisae* culture with colony count at 10^8 cfu/ml as described below and fermentation was carried out at pH4.5, with shaking at 200rpm and various temperatures degrees (20, 30, and 40°C). Ethanol content in fermented sample was estimated after 3 days of incubation as described below

Fermentation

Bioethanol fermentation was conducted in liquid state fermentation. *S. cerevisae* was used for fermentation. The yeast inoculum was prepared in YEPD broth. A loopful of twenty four hours old culture was inoculated and incubated at 30 °C on rotary shaker (200 rpm) for twenty four hours. The initial yeast count in fermentation sample was 10^8 cfu /ml. The production medium used for ethanol fermentation was composed of:

glucose (sugars solution obtained from saccharified starch), 0.1% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 0.05% $MgSO_4.7H_2O$ and 0.1% yeast extract, pH was adjusted to pH3.5. The medium is introduced in 250 mL capacity flasks containing 100 mL of the fermentation medium. This inoculum was used at 10 % (10^8 cfu/ml) to inoculate the hydrolyzate from the pretreated potato waste mash samples. All experiments were incubated as described above. The ethanol content was measured after fermentation (Caputi *et al.,* 1968)**.**

Statistical analysis

Data were calculated as mean ± standard deviation (SD) and analyzed using analysis of variance (ANOVA). Probability of 0.05 or less was considered significant. The statistical package of Costat Program (1986) was used for all chemometric calculations.

Results and Discussion

The most widely used screening method for starch hydrolyzing organisms is the so called "clear zone" method. The extracellular hydrolyzing enzymes secreted by the target organism degrade the suspended starch in the agar medium into water soluble products thereby producing zones of clearance around the colony. The main advantage of this test is that it is generally fast, cheap and simple, and allows the simultaneous performance of a great number of parallel tests (Belal, 2003; Belal and El-Mahrouk, 2010; Belal and Khalafalla, 2011; Belal, 2013b; Belal *et al.,* 2014).

From the microbial source (soil) a total of 50 morphologically different starch hydrolyzing isolates were obtained. Among 50 bacterial isolates, one bacterial isolate designated as E19 exhibited the highest starch degradation ability (widest clear zone) comparing with the other isolates. The results were compared with those obtained with noninoculated medium incubated in the respective media. E19 was found to be the most efficient starch degrading bacterial isolate and degraded starch completely on agar plates after 4 days, therefore, this isolate was selected for further investigations. Screening of purified cultures was done on medium supplemented with 1% starch the cultures growing in cultivation medium were flooded with iodine solution and the zone of hydrolysis were obtained in the plates showing starch hydrolysis. Similar method has been used earlier by Khan and Briscoe (2011) and Belal *et al.* (2014).

Identification of the efficient of starch degrading isolate

Results in figure 1 showed that this bacterial strain (E19) was identified according to morphological, physiological as well as using analysis of 16S rDNA. This efficient starch hydrolyzing strain (E19) was gram-postive, endospore forming, motile, and rods. According to the 16S rDNA analysis, the phylogenetic tree of the starch degrading bacteria E19 and related bacterial species based on the 16S rDNA sequence is provided in figure 1. It can be clearly seen that the *Bacillus* sp. E19 as starch hydrolyzing bacteria was included in the genus *Bacillus* and closely related to the species *subtilis*. It showed the highest sequence similarities with *Bacillus subtilis* strain RhAn18 (100 %), respectively (Figure 1). Among bacteria, *Bacillus* sp. is widely used for thermostable α-amylase production to meet industrial needs. *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, *B. amyloliquefaciens* and *B. mojavensis* are known to be good producers of α-amylase and most of these have been widely used for commercial production of the enzyme for various applications (Hagihara *et al.,* 2001;

Jadhav *et al.,* 2013; Belal *et al.,* 2014)**.** This strain was used in the subsequent experiments to determine it degradability for potato waste powder under solid state fermentation. The starch potato waste mash content was 19.8%.

Effect of different pH and temperature on *B. subtilis* biomass and its amylase production

The influence of pH on *B. subtilis* growth and its amylase production in solid state fermentation (SSF) of potato waste powder is shown in table 1. The optimum pH value for *B. subtilis* growth and potato waste powder degradation was found to be 6.5. For pH, enzyme activity increased from its lowest value at pH 6 to reach its maximum at pH 6.5 before subsequently decreasing at pH7, 7.5 and 8. The degradation efficiency of *B. subtilis* was compared across a wide range of pH (6–8). The highest *B. subtilis* biomass and its amylase activity (U/ml/min) with were achieved at pH 6.5. Nevertheless, the pH of the medium is one of the main parameters which affect enzyme yields because pH changes can affect the stability of amylases in the medium**.** Most of the starch degrading bacterial strain revealed a pH range between 6.0 and 7.0 for normal growth and enzyme activity (Gupta *et al.,* 2003; Belal *et al.,* 2014).

The effect of different temperatures on *B. subtilis* growth and its amylase activity (U/ml/min) in SSF of potato waste powder at pH 6.5 is shown in table 2. Degradation of potato waste powder occurred at 20, 30 and 40 °C. The highest bacterial biomass and amylase production was achieved at 30°C and least bacterial growth and amylase production at 20 and 40°C, respectively. A decrease or increase in incubation temperature caused a decrease in enzyme production by *Bacillus subtilis*. The results were compared with those obtained with control incubated in the respective media. This could be attributed to the ability of the organism to survive a range of temperatures, a feature yet to be investigated since the effect of temperature on amylase production is related to the growth of the microbial strain (Belal *et al.,* 2014)**.**

Effect of the inoculum size and incubation period on *B. subtilis* growth and its amylase production

Varying responses were obtained when different inoculum sizes were used for *B. subtilis* growth and its amylase production in SSF of potato waste powder (Table 3). *B. subtilis* growth and it amylase activity increased gradually from 5% till 20% inoculum concentration. The best inoculum size was 20%. The obtained results exhibited also that no relationship between increasing inoculum size and amylase production, where the *B. subtilis* biomass increased with decreasing in its amylase production at inoculum concentration 25% and this may be due to the starch content in potato waste powder was degraded completely. The results were compared with those obtained with control medium incubated in the respective media. Our results are in agreement with Saxena and Singh (2011). Bayasl *et al.* (2008) have also reported 20% inoculum size as optimum in SSF of wheat bran in amylase production. But in this particular case, rapid growth of the isolate could have caused the inoculum to increase rapidly, thereby causing the availability of substrate to become the limiting factor in enzyme production instead of the varying inoculum level (Aullybux and Puchooa, 2013).

A series of experiments were carried out to study the behaviour for *B. subtilis* growth and its amylase production in SSF of potato

waste powder. Results in figure 2 show a maximum of growth was obtained after 4 days. The obtained results showed also that the amylase formation started when the organism grew on the medium. The highest of amylase activity exhibited after 4 days from cultivation and after that the amylase activity decreased. Amylase excretion coincided with increase in the specific growth rates of the cultures and potato waste wastes powder degradation. The starch content in potato waste flour was degraded completely after 4 days. Enzyme production by *B. subtilis* is directly correlated to the time period of incubation. Thus optimum time of enzyme synthesis was to be 4 days after inoculation and this may be due to the solid state fermentation technique increased the growth phase time of *B. subtilis*. The results were compared with those obtained with noninoculated medium incubated in the respective media. The abiotic loss in control treatment was 0.21%. Optimization of culture conditions is very important for maximum microbial growth and enzyme production by microbial strains and is a prime step in fermentation technology (Kathiresan and Manivannan, 2006**).**

Bioethanol production from potato waste powder hydrolyzate by *Saccharomyces cerevisae*

Results in table 4 exhibited that the obtained reducing sugars resulted from potato wastes powder pretreatment with *B. subtilis* and *B. subtilis* amylase. The individual treatment of the treatments resulted in high yield of reducing sugars. As shown in table 4, the present study showed also that, the general trend of potato wastes powder bioconversion with *B. subtilis* amylase was more than the general trend by *B. subtilis*. An initial increasing trend of sugar formation was observed when more of potato wastes powder substrate was degraded with a fixed enzyme concentration. Therefore, crude *B. subtilis* amylase was selected to optimize of bioethanol production from potato waste powder in the present study. The reducing sugars were 0.22% in noninoculated medium due to abiotic loss. The bioethanol in noninoculated medium recoded 0%. There are some possible reasons for these differences, including the strain of *S. cerevisiae* used, biochemical composition of the substrate, fermentation system and the condition under which the fermentation took place (Chen *et al.*, 2007).

Effect of incubation period on bioethanol production from hydrolyzate potato waste powder by *S. cerevisiae*

The concentration of ethanol increased with the increase of fermentation time. The maximum ethanol production (8.9%) was obtained after 3 days of incubation (Table 5). The ethanol production increased gradually till the third day and after that it decreased. The reduction of ethanol production may be due to the toxic effect on growth of yeast. Our results, is similar with Afifi *et al.* (2011) and Belal *et al.* (2014), they reported that maximum ethanol concentration was obtained after 72 h.

Effect of inoculum size on bioethanol production

The obtained results in table 6 show difference among the inoculum size tested (5, 10, 15 and 20 %) on ethanol production. The maximum ethanol concentration was produced by *S. cerevisiae* with inoculum size of 10 %. Sugar utilization and highest ethanol yield that obtained with an initial inoculum of 10%. Ethanol production was decreased with inoculum size of 15 and 20%. Afifi *et al.* (2011) produced maximum ethanol from industrial solid potato wastes when inoculated with 10% (v/w) inoculum size of *S. cerevisiae*.

Table.1 Effect of different pH on *B. subtilis* growth and its amylase production

Treatments	*B. subtilis* growth and its amylase activity (U/ml/min) at different pH									
	pH6		pH6.5		pH7		pH7.5		pH8	
	amylase activity (U/ml/min)	Log (cfu/ml)	amylase activity (U/ml/min)	Log (cfu/ml)	amylase activity (U/ml/min)	Log (cfu/ml)	amylase activity (U/ml/min)	Log (cfu/ml)	amylase activity (U/ml/min)	Log (cfu/ml)
B. subtilis + Potato waste powder	470 ± 0.12	2.9 ± 0.22	1150 ± 0.21	8.9 ± 0.71	870 ± 0.58	6.2 ± 0.31	710 ± 0.61	5.1 ± 0.51	620 ± 0.11	3.9 ± 0.24
Potato waste powder	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table.2 Effect of different temperature on *B. subtilis* growth and its amylase

Treatments	*B. subtilis* biomass and amylase activity (U/ml/min) at different temperature (°C)					
	20 °C		30 °C		40 °C	
	Amylase activity (U/ml/min)	Log (cfu/ml)	Amylase activity (U/ml/min)	Log (cfu/ml)	Amylase activity (U/ml/min)	Log (cfu/ml)
B. subtilis + Potato waste powder	510 ± 0.33	4.2 ± 0.31	1280 ± 0.21	10.2 ± 0.33	270 ± 0.22	3.8 ± 0.31
Potato waste powder	0.0	0.0	0.0	0.0	0.0	0.0

Table.3 Effect of the inoculum size on *B. subtilis* growth and its amylase production

Treatments	*B. subtilis* biomass and amylase activity (U/ml/min) at different inoculum size (%)									
	5%		10%		15%		20%		25%	
	Amylase activity (U/ml/min)	Log (cfu/ml)	Amylase activity (U/ml/min)	Log (cfu/ml)	Amylase activity (U/ml/min)	Log (cfu/ml)	Amylase activity (U/ml/min)	Log (cfu/ml)	Amylase activity (U/ml/min)	Log (cfu/ml)
B. subtilis + Potato waste powder	620 ± 0.22	7.9 ± 0.51	890 ± 0.34	8.8 ± 0.71	1190 ± 0.41	9.9 ± 0.11	1610 ± 0.33	10.4 ± 0.32	1260 ± 0.61	10.9 ± 0.51
Potato waste powder	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table.4 Bioethanol production from potato waste powder hydrolyzate (by *B. subtilis and B. subtilis* amylase) fermented by *S. cerevisae*

B. subtilis and its amylase / *Reducing sugars and ethanol*	Reducing sugars and ethanol production by S. cerevisae	
	B. subtilis	*B. subtilis* amylase
Reducing sugars (w/v)	17.6 ± 0.41	18.4 ± 0.11
Ethanol (v/v)	8.3 ± 0.33	8.9± 0.21

Table.5 Effect of incubation period on bioethanol production from pretreated potato wastes powder by *B. subtilis* amylase

Reducing sugars and incubation period / Ethanol	Bioethanol production at different incubation period and reducing sugars (18.4 w/v)				
	Incubation period (day)				
	1	2	3	4	5
Ethanol (%)	3.1 ± 0.25	6.4 ± 0.43	9.01 ± 0.13	4.6 ± 0.31	2.32 ± 0.3
Control (untreated)	0.0	0.0	0.0	0.0	0.0

Table.6 Effect of inoculum size on bioethanol production from hydrolyzate potato waste powder by *S. cerevisiae*

Reducing sugars and inoculum size / Ethanol	Bioethanol production with inoculum size and reducing sugars (18.4 w/v)			
	Inoculum size (%)			
	5	10	15	20
Ethanol (%)	5.2 ±0.27	8.9 ±0.12	6.6 ± 0.45	6.2±0.51
Control (untreated)	0.0	0.0	0.0	0.0

Table.7 Effect of pH on bioethanol production from hydrolyzate solid potato waste by *S. cerevisiae*

Reducing sugars and pH / Ethanol	% Bioethanol production at different pH and reducing sugars (18.4 w/v)						
	pH						
	4	4.5	5	5.5	6	6.5	7
% Ethanol	5.6 ± 0.31	9 ± 0.1	4.5 ± 0.22	4.1 ± 0.24	3.4 ± 0.41	2.4 ± 0.33	1.9 ± 0.12
Control (untreated)	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table.8 Effect of temperature on bioethanol production from hydrolyzate potato waste powder by *S. cerevisiae*

Reducing sugars and temperature / Ethanol	% Bioethanol production at different temperature and reducing sugars (18.4 w/v)		
	Temperature (°C)		
	20	30	40
% Ethanol	4.1 ± 0.31	9.01 ± 0.11	3.2 ± 0.22
Control (untreated)	0.0	0.0	0.0

Figure.1 Phylogenetic tree showing the diversity of bacterial 16 S gene sequences from *Bacillus subtilis* (E19). Phylogenetic trees were generated using parsimony, neighbor-joining and maximum likelihood analysis. The scale bar represents 0.025 substitutions per nucleotide position

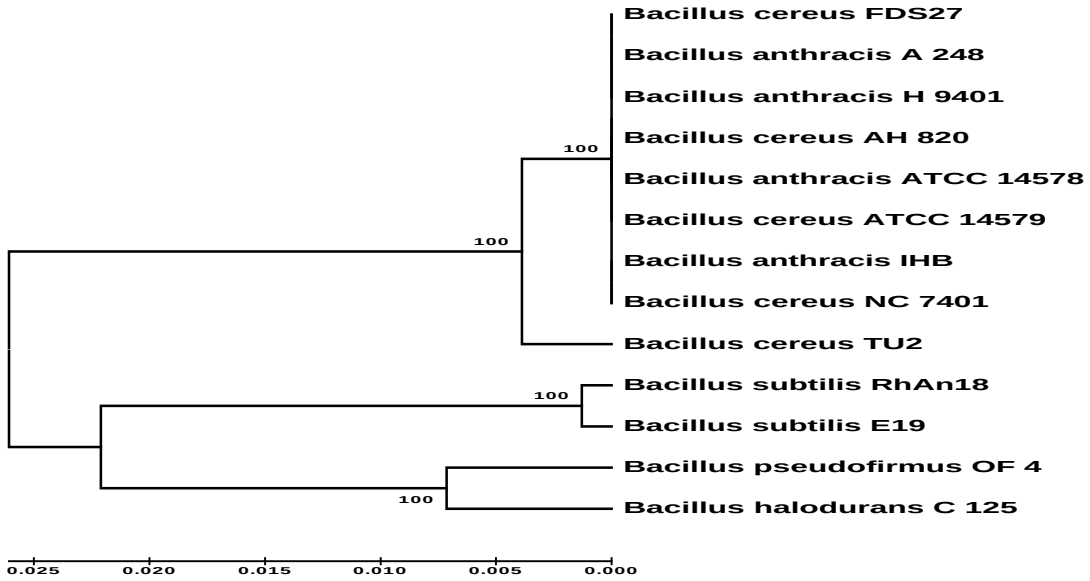
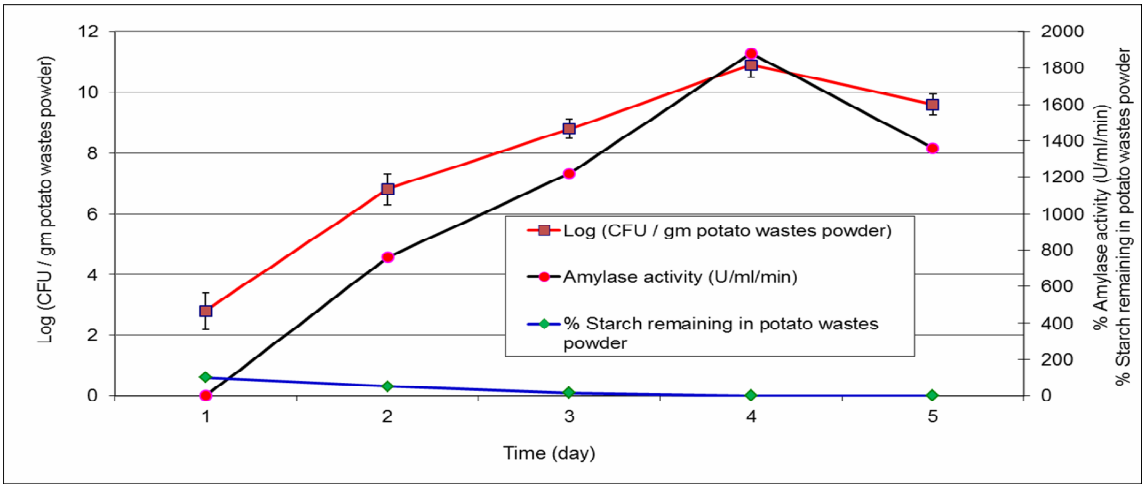


Figure.2 Effect of incubation period on *B. subtilis* growth and its amylase production



Effect of pH and temperature on bioethanol production from hydrolyzate solid potato waste by *S. cerevisiae*

The influence of pH on bioethanol production from hydrolyzate solid potato waste (reducing sugars) is shown in table 7. Generally, the optimum pH was 4.5 for bioethanol production by *S. cerevisiae*. The maximum of bioethanol production was recorded at pH4.5 and decreased marginally above this value. Bioethanol production was at quite wide pH range (from 4 to 7). Therefore, it can expect that pH is one of the most important factors for bioethanol production by *S. cerevisiae*. Roukas (1994) studied the effect of pH on ethanol production from carob pod by *S. cerevisiae* and found that the maximum ethanol concentration, ethanol yield, and fermentation efficiency were obtained at pH 4.5.

The effect of different temperatures on bioethanol production is shown in table 8. A temperature 30 °C appears to be the optimum for bioethanol production. The ethanol yield decreased at temperature values lower or higher than 30 °C. The ethanol concentration increased with the increase in fermentation temperature from 20 to 30 °C and decreased gradually between 30 and 40 °C. This is in agreement with work reported by other workers **(**Arifa *et al.,* 2010 and Belal *et al.,* 2014).

According to the results, it could be concluded that potato can be an attractive feedstock for the bioethanol production, especially in Egypt, where waste potato by-products are obtained from potato cultivars and also due to poor storage facility. Bioconversion offers a cheap and safe method of not only disposing the starchy wastes by solid state fermentation (SSF) of potato wastes powder under optimum conditions, but also it has the potential to convert starchy wastes into usable forms such as reducing sugars that could be used for ethanol production. These results help to produce cheap clean energy and reduce the environmental pollution which caused by starch waste.

References

Adarsha, R., Asha, D.L., Balaji, R.R. 2010. Production of bio-ethanol from *Pectobacterium carotovorum* induced soft rotten potatoes. *Afr. J. Microbiol. Res.*, 4(12): 1340–1342.

Afifi, M.M., Abd El-Ghany, T.M., Al Abboud, M.A., Taha, T.M., Ghaleb, K.E. 2011. Biorefinery of industrial potato wastes to ethanol by solid state fermentation. *Res. J. Agric. Biol. Sci.,* 7(1): 126–134.

Arifa, T., Aftab, M., Farasat, T. 2010. Effect of cultural conditions on ethanol production by locally isolated *Saccharomyces cereviviae* BIO-07. *J. App. Pharm.,* 3(2): 72–78.

Aullybux, A.A., Puchooa, D. 2013. α-Amylase Production on Low-Cost Substrates by *Naxibacter* sp. isolated from Mauritian Soils. *Brit. Microb. Res. J.,* 3(4): 478–491.

Baysal, Z., Uyar, F., Dogru, M., Alkan, H. 2008. Production of extracellular alkaline α amylase by SSF with a newly isolated *Bacillus* sp. *Prep. Biochem. Biotechnol.,* 38: 184–190.

Belal, E.B. 2013a. Bioethanol production from rice straw residues. *Braz. J. Microb.*, 44(1): 225–234.

Belal, E.B. 2013b. Biodegradation of aliphatic - aromatic copolyester under thermophilic conditions. Res. *J. Environ. Earth Sci.,* 5(11): 677–690.

Belal, E.B., El-Khateeb, N.M., Teiba, I.I., Elzahabi, H.M. 2014. Bioethanol

production from solid Potato Wastes. *Egypt. J. plant Prot. Res.,* 2(4): 2314–5951.

Belal, E.B., El-Mahrouk**,** M.E. 2010. Solid – state fermentation of rice straw residues for its use as growing medium in ornamental nurseries. *Acta Astronautica,* 67: 1081–1089.

Belal, E.B., Khalafalla, M.M.E. 2011. Biodegradation of *Panicum repens* residues by *Pleurotus ostreatus* for its use as a non conventional feedstuff in diets of *Oreochromis niloticus*. *Afr. J. Microbiol. Res.,* 5(19): 3038–3050.

Belal, E.B.A. 2003. Investigation on the biodegradation of polyesters by isolated mesophilic microbes. Dissertation, Technical University Braunschweig, Germany.

Caputi, A.J., Ueda, M., Brown, T. 1968. Spectrophotometric determination of ethanol in wine. *Am. J. Enol. Vitic.* 19:160–165.

Chen, H.Z., Xu, J., Li, Z.H. 2007. Temperature cycling to improve the ethanol production with solid-state simultaneous saccharification and fermentation. *Appl. Biochem. Microbiol.,* 43: 57–60.

Claudio, T. S., Whitney, A.M., Mayer, L.W., Morey, R., Steigerwalt, A., Boras, A., Weyant, R.S., Popovic T. 2002. Sequencing of 16S rRNA Gene: A Rapid Tool for Identification of *Bacillus anthracis*. *Emerg. Infect. Dis.,* 8(10): 1117–1123.

Costat Program 1986. Version 2, Cohort Software, Minneapolis, MN, USA.

Duhan, J.S., Kumar, A., Tanwar S.K. 2013. Bioethanol production from starchy part of tuberous plant (potato) using *Saccharomyces cerevisiae* MTCC–170. *Afr. J. Microb. Res.,* 7(46): 5253–5260.

Fadel, M. 2000. Alcohol production from potato industry starchy waste. *Egypt. J. Microbiol.,* 35(3): 273–287.

FAO (Food and Agriculture Organization of the United Nations) 2014. Crop Production, Egypt, 2011 data.

Grassi, G. 1999. Modern bioenergy in the European Union renewable energy, 16: 985–990.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V.K., Chauhan, B. 2003. Microbial α-amylases: a biotechnological perspective. *Proc. Biochem.,* 38:1599–616.

Hagihara, H., Igarashi, K., Hayashi, Y., Endo, K., Ikawa-Kitayama, K., Ozaki, K., Kawai S., Ho, S. 2001. Novel α amylase that is highly resistant to chelating reagents and chemical oxidants from the alkaliphilic *Bacillus* isolate KSM.K.38. *Appl. Environ. Microbiol.,* 67: 1744–1750.

Jadhav, S.A., Kataria, P.K., Bhise, K.K., Chougule S.A. 2013. Amylase production from potato and banana peel waste. *Int. J. Curr. Microbiol. App. Sci.,* 2(11): 410–414.

Kathiresan, K., Manivannan, S. 2006. α-Amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil. *Afr. J. Biotech.,* 5(10): 829–832.

Khan, A.J., Briscoe, S. 2011. A study on partial purification and characterization of extracellular alkaline amylases from *Bacillus megaterium* by state fermentation. *Intern. J. Appl. Biol. Pharm. Tech.,* 2(3): 37–46.

Khan, R.A., Nawaz, A., Ahmed, M., Khan, M.R., Azam, F.D.N., Ullah, S., Sadullah F., Ahmad, A., Shah, M.S., Khan, N.2012. Production of bioethanol through enzymatic

hydrolysis of potato. *Afr. J. Biotech.,* 11(25): 6739–6743.

Lowry, O.H., Rsebrough, N.J., Farr, A.L., Rundal, R.L. 1951. Protein measurements with the folin phenol reagent. *J. Biol. Chem.,* 193: 265–275.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Annl. Chem.,* 31: 426–428.

Nanganuru, H.Y., Korrapati, A.N., Mutyala, S. 2012. Studies on the production of α- amylase by *Bacillus subtilis*. *IOSR J. Eng.,* 2(5): 1053–1055.

Nei, M., Kumar, S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Pandey, A., Nigam, P., Soccol, C.R., Soccol, V.T., Singh, D., Mohan, R. 2000. Advances in microbial amylases. *Biotechnol. Appl. Biochem.,* 31: 135–152.

Parry, J.M., Turnbull, P.C.B., Gibson, J.R. 1983. A colour atlas of *Bacillus* species, Wolfe Medical Publications Ltd.

Rameshkumar, A., Sivasudha, T. 2011. Optimization of nutritional constitute for enhanced alpha amylase production using by solid state fermentation technology. *Int. J. Microb. Res.,* 2 (2): 143–148.

Roukas, T. 1994. Solid-state fermentation of carob pods for ethanol production. *Appl. Microbiol. Biotechnol.,* 41: 296–301.

Rzhetsky A., Nei, M. 1992. A simple method for estimating and testing minimum evolution trees. *Mol. Biol. Evol.,* 9: 945–967.

Saitou, N., Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.,* 4:406–425.

Saxena, R., Singh, R. 2011. Amylase production by solid-state fermentation of agro-industrial waste using *Bacillus* sp. *Braz. J. Microbiol.,* 42(4): 1334–1342.

Sehsah, E.E, Belal, E.B., Abu sheisha, R.R., Ellawaty, A. 2014. Bio-ethanol application as an alternative fuel in farm machine. *Misr. J. Ag. Eng.,* 31(5): 1232–1241.

Sodhi, H.K., Sharma, K., Gupta, J.K., Soni, S.K. 2005. Production of a thermostable α amylase from *Bacillius* sp. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production. *Proc. Biochem.,* 40: 525–534.

Tamura, K., Nei, M., Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. (USA),* 101: 11030–11035.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0**.** *Mol. Biol. Evol.,* 30: 2725–2729.

Yamada, S., Shinomiya, N., Ohba, K., Sekikawa, M., Oda. Y. 2009. Enzymatic hydrolysis and ethanol fermentation of by-products from potato processing plant. *Food Sci. Technol. Res.,* 15(6): 653–658.