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Production of amylases from *Bacillus amyloliquefaciens* under submerged fermentation using some agro-industrial by-products

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Abstract Thirty-one bacterial isolates out of 133 isolates, were obtained from rhizosphere of Egyptian clover plants, and had variant capability for starch degradation on starch agar medium. The isolate E109 was the most potent being 72.5 U ml^{-1} and 2.5 for amylase activity and starch hydrolysis ratio (SHR), respectively, at 50 °C. The potent isolate E109 was identified based on phenotypic characteristics, phylogenetic positions based on 16S rRNA gene analysis and base sequences (submitted to NCBI Gen Bank). 16S rRNA gene analysis confirmed that this isolate belonged to the genus *Bacillus* and it was most closely related to *B. amyloliquefaciens* (95% similarity). For the production of amylases, nine agro-industrial residues were added as carbon sources to the basal medium. The medium supplemented with potato starchy waste as the sole carbon source enhanced the enzyme activity more than soluble starch as control for α, β and γ amylases activity, as it increased by *B. amyloliquefaciens* about 1.26 & 4 and 8-fold, respectively after 48 h at 50 °C using rotary shaker at 150 rpm. *B. amyloliquefaciens* gave the maximum values of α, β and γ amylases activity on medium supplemented with 2% potato starchy waste after 30, 30 & 36 h of fermentation periods at 50 °C using shake flasks technique as a batch culture. These values were 155.2 U ml^{-1} (R^2 = 0.93), 1.0 U ml^{-1} (R^2 = 0.94) and 2.4 U ml^{-1} (R^2 = 0.95), respectively. It could be stated that productive medium supplemented with 2% potato starchy waste as a low price substrate could be more favorable than basal medium containing 1% starch for amylases production in submerged fermentation, as it increased α, β and γ amylase activity by 1.98, 7.69 and 12-fold than that produced in basal medium (control), respectively.

Introduction

Enzymes are defined as biocatalysts protein in nature, produced by living cells to bring about specific biochemical reactions, generally forming parts of the metabolic processes of the cell. Enzymes are highly specific in their action on

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substrates. The production of amylases is overshadowing all other enzymes; hence, amylases account 65% of enzyme market in world (van der Maarel et al., 2002; Balkan and Figen, 2007). α-amylase is an endo-acting enzyme and hydrolyzes linkages in a random fashion that hydrolyzes α-1,4 bonds and bypass α-1, 6 linkages and leads to the formation of linear and branched oligosaccharides and limit dextrins. β-amylase is an exo-acting enzyme that attacks the substrate from the non-reducing end and hydrolyzes α-1, 4 and cannot bypass α-1, 6 linkages thus producing oligosaccharide maltose (2 units of adjacent glucose) as a major end product. γ-Amylase (glucoamylase) is an exo-acting enzyme that attacks the substrate from the non-reducing end and hydrolyzes α-1, 4 and α-1, 6 linkages thus producing monosaccharides (1 unit of glucose) as a major end product (Gupta et al., 2002).

Amylases are used commercially for starch liquefaction, paper, desizing of textile fabrics, in preparing starch coatings of paints, in removing wall paper, food in the brewing industry, sugar induction by production of sugar syrups from starch which consist of glucose, maltose and higher oligosaccharides, pharmaceutical and in preparing cold water dispersible laundry starches. To meet the demands of these industries low cost medium is required for the production of amylases (Balkan and Figen, 2007). Nowadays the potential of using microorganisms as a biological source of industrially economic enzymes has stimulated interest in the exploitation of extracellular enzymatic activity in several microorganisms. Amylases can be obtained from several sources such as plant, animal and microbes such as bacteria and fungi (Murakami et al., 2008). The microbial source of amylases is preferred to other sources because of its plasticity and vast availability. Until now all commercial enzymes have been derived from cultivated bacteria or fungi. Bacterial amylases are generally preferred for starch processing. Among bacteria, *Bacillus* species such as *B. subtilis*, *B. stearothermophilus*, *B. macerans*, *B. megaterium* and *B. amyloliquefaciens* were the best producers of thermostable α-amylase using submerged fermentation and these have been widely used for commercial production of the enzyme for various applications (Enhasy, 2007; Božić et al., 2011), *Clostridium thermosacharolyticum*, *Cl. thermohydrosulfuricum* and *Pseudomonas* sp. (Mrudula et al., 2011).

Biosynthesis of amylases was performed on agro-industrial wastes and by-products such as starchy materials to solve pollution problems and obtain a low cost medium (Haq et al., 2005; Djekrif-Dakhmouche et al., 2006; Anto et al., 2006; Mukherjee et al., 2009). Rice husk, wheat bran and potato starchy waste were used as a low cost carbon substrate for amylase activity by *B. subtilis* (Baysal et al., 2003; Shukla and Kar, 2006; Asgher et al., 2007).

The objective of this study was to investigate the agro-industrial residues as an alternative carbon source to produce amylases by Egyptian local bacteria in order to reduce environmental pollution and product cost.

Materials and methods

Samples collection from soil environment

Rhizosphere samples were collected from the fertile fields planted with Egyptian clover (*Trifolium alexandrinum*), in Qalyubia governorate. Soil samples were taken from 3 to 5 cm depth after removing 5 cm from the ground surface. These samples were collected into sterilized plastic bags and stored in ice-boxes during their transport to the laboratory. In the laboratory all samples were kept refrigerated until isolation.

Media used

Medium (1): Nutrient agar medium (DIFCO Manual, 1984) was used for maintenance and preservation of bacteria. Medium (2): Starch agar medium (Madigan et al., 2011) was used for isolation of starch-degrading bacteria. Its composition was as follows (gl^{-1}): soluble starch, 10; KNO_3,0.5; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; $FeCl_3$, traces; agar, 15 and adjusted to pH 7.0. *Starch broth medium was the same as starch agar medium without adding agar.

Isolation and screening of the amylolytic bacteria

Ten gram representative soil sample was suspended in 90 ml of sterile tap water and shaken thoroughly for 10 min. Starch-degrading microorganisms were isolated from collected samples by the soil dilution plate technique using starch agar medium according to Clark et al. (1958). Serial dilutions up to 10^{-7} of each soil sample were prepared using sterilized water. Suitable dilutions were plated (in triplicates) on the above solid medium. The poured plates were incubated at 30 and 50 °C for 48 h. After 48 h plates were flooded with 1% Lugol's iodine reagent for 20 min. and washed with water to remove the excess color. The clear halo-zone around colonies were measured to calculate starch hydrolysis ratio (SHR) according to Thippeswamy et al. (2006) and Bahadure et al. (2010). The selected isolates were preserved on agar slant for further use.

Submerged fermentation process

It was carried out in 250 ml plugged Erlenmeyer flasks, each containing 100 ml sterile starch broth medium and inoculated with 1% of standard inoculum (2.3×10^6 CFU ml^{-1}) for the tested bacterial isolate which incubated at 50 °C on rotary shaker at 150 rpm for 48 h. The fermented medium was centrifuged at 10,000 rpm for 10 min in order to determine periodically the cell dry weight and amylases activity in the precipitate and supernatant, respectively. All the experiments were carried out at least in triplicate (Fossi et al., 2011).

Preparation of agro-industrial residues

Nine agriculture by-products namely; wheat bran, wheat straw, rice bran, rice straw, rice husk, broken rice, maize starch, potato starchy waste and corncobs were washed with cold water and subsequently with warm water to get rid of dust and impurities. The washed substrates were then dried at 50 °C overnight in order to get constant weight. The dried substrates were grinded in a laboratory grinder and a particle size of 5 mm was selected by sieving to remove the large particles and used for further studies (Singh and Rani, 2014).

Effect of agro-industrial residues as starch substrates on enzymes activity

In submerged fermentation, growth and enzymes activity were studied on different starch substrates as carbon sources during different fermentation periods. The appropriate carbon source was selected by replacing the original carbon source of the used medium with equivalent carbon amount of each of the tested carbon sources (wheat bran, wheat straw, rice bran, rice straw, rice husk, broken rice, maize starch, potato starchy waste and corncob). Different potato starchy waste concentrations ranging between 0.5% and 2.5% were used to select the proper treatment for enzyme production by the tested strain on the productive medium.

Phenotypic and genotypic identification

Identification of selected isolate was carried out according to their morphological (Gram and endospore staining were observed under light microscope) and biochemical tests (catalase, starch hydrolysis, gelatin hydrolysis, casein hydrolysis, indole production and Voges–Proskauer test) based on Bergey's Manual of Systematic Bacteriology 2nd ed. (Niall and Paul, 2009). It was then confirmed with 16S rRNA sequencing, pure cultures of the target bacteria were grown in nutrient broth medium on a rotary shaker (150 rpm) at 30 °C for 24 h for the isolation of genomic DNA using the method described by Hiney and colleagues (Yadav et al., 2009). Amplification of 16S rDNA by PCR was done using universal bacterial primers (27F) forward F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and (1492R) reverse R (5′- GGT TAC CTT GTT ACG ACT T-3′) described by Raji et al. (2008). Amplification was carried out in a 50 µl reaction volume. The thermal cycle (PCR) steps were applied as follows: 5 min initial denaturation at 95 °C, followed by 30 cycles of 1 min denaturation at 95 °C, 1 min primer annealing at 55 °C, 1 min extension at 72 °C and a final 10 min extension at 72 °C. The amplified DNA fragment was separated on 1% (w/v) agarose gel electrophoresis, eluted and purified using the Qiaquick gel extraction kit (Qiagen, Germany) following the manufacturer's protocol (Nimnoi et al., 2010). The purified PCR product was sequenced using the Big-Dye terminator kit ABI 310 Genetic Analyzer (Applied Biosystems, USA). Sequence data of partial 16S rDNA were aligned and analyzed for finding the closest homologous microbes. The unknown query 16S rRNA nucleotide sequence was compared to nucleotide databases using BLASTN program that is available from the National Center for Biotechnology Information (NCBI, 2014) and retrieved from Gene Bank database. Then multiple sequence alignment was developed for these homologous sequences using the algorithm described in Clustal Omega. A phylogenetic tree was then drawn using the neighbor joining method.

Analytical procedures

Cell dry weight was determined by washing the pellets with distilled water in order to remove the starchy material attached to the pellet where the cellular mass was suspended and homogenized with 20 ml of distilled water, following centrifugation at 10,000 rpm for 10 min at 4 °C, and the supernatant was put aside during three cycles. Finally, the biomass was suspended in 1 ml of distilled water. The suspension was added to preweighted caps and dried in an oven at 60 °C for 1–2 days to a constant weight for the measurement of cell dry weight (gl^{-1}) (Tamilarasan et al., 2012).

Alpha amylase activity was assayed using starch–iodine method described by Sudharhsan et al. (2007). The activity was estimated using 0.5 ml of 1% soluble starch (Sigma S-2630) gelatinized in water (15 min; 100 °C; continuous mixing) soaked in 0.5 ml 0.1 M phosphate buffer (pH 7.0), 1 ml of crude enzyme was mixed and incubated at 60 °C for 30 min, where the assayed enzymes were most active, and 1 ml of 1 M HCl was added to stop the enzymatic reaction, followed by the addition of 1 ml of iodine reagent (5 mM I_2 and 5 mM KI). Following color development the absorbance at 620 nm was measured using spectrophotometer (Unico S2100 series UV/Vis). Starch–iodine assay is defined as the disappearance of an average of 1 mg of iodine binding starch material per min in the assay reaction. U ml^{-1} was calculated using the following formula:

$$\text{U ml}^{-1} = (A_{620\ \text{nm}}\ \text{control} - A_{620\ \text{nm}}\ \text{sample})/(A_{620\ \text{nm}}/\text{mg starch})/30\ \text{min}/1\ \text{ml}/\text{dilution factor}.$$

where $A_{620\ \text{nm}}$ control is the absorbance obtained from the starch without the addition of enzyme, $A_{620\ \text{nm}}$ sample is the absorbance for the starch digested with enzyme, and $A_{620\ \text{nm}}$/mg starch is the absorbance for 1 mg of starch as derived from the standard curve (Yoo et al., 1987; Pfueller and Elliot, 1999; Xiao et al., 2005).

Beta or gamma amylases activity was determined by measuring the amount of glucose liberated from starch (Bryjak, 2003). A 0.5 ml of 1% soluble starch (Sigma S-2630) gelatinized in water (15 min; 100 °C; continuous mixing), 0.5 ml 0.1 M acetate buffer (pH 4.8 or 4.5) and 0.5 ml sample of crude enzyme were mixed and incubated for 3 min at 45 or 55 °C, respectively. The glucose released was measured by the glucose oxidase peroxidase kits from (BIO-ADWIC) EL NASR PHARMACEUTICAL CHEMICALS Co. (Egypt). Beta or gamma amylases activity was calculated from the amount of reduced sugar produced, by the following formula recommended by Haq et al. (2003) and Karnwal and Nigam (2013):

U ml^{-1} = (amount of reducing sugar × dilution factor)/(1000 × MW of glucose (180.2) × time × enzyme volume). One unit (U) of beta and gamma amylases is that amount of enzyme which liberates 1 µmole of glucose in 1 min.

Parameters calculation

Specific growth rate (μ) (h^{-1}) and doubling time (t_d) (h), multiplication rate (MR) and number of generations (N) were calculated using the following equations according to Doelle (1975). The following formulas were used to calculate these parameters: specific growth rate per hour $(\mu) = (\ln X - \ln X_0)(t - t_0)^{-1}$, doubling time $(t_d) = \ln 2\ (\mu)^{-1}$, multiplication rate (MR) = $1(t_d)^{-1}$ and number of generation $(N) = (t - t_0)(t_d)^{-1}$.

Starch hydrolysis ratio (SHR) was calculated using the following equation

SHR = Clear halo zone diameter (mm)/colony growth diameter (mm) (Thippeswamy et al., 2006; Bahadure et al., 2010).

Statistical analysis

The collected data were statistically analyzed using IBM® SPSS® Statistics software (2011) and the correlation coefficient was analyzed with Microsoft Office Excel 2013.

Results and discussion

Isolation and selection of starch degrading bacteria

Among 133 bacterial isolates, thirty-one were amylolytic and could be classified into three categories (high, moderate and weak) according to the degree of starch hydrolysis. Fig. 1 illustrates their percentage distribution which is expressed as diameter of clear zone (mm). Thirty-one amylolytic isolates could be also classified into two types according to their optimum growth temperature. Ten isolates were mesophilic whereas, twenty-one isolates grew at 50–60 °C better than 30 °C.

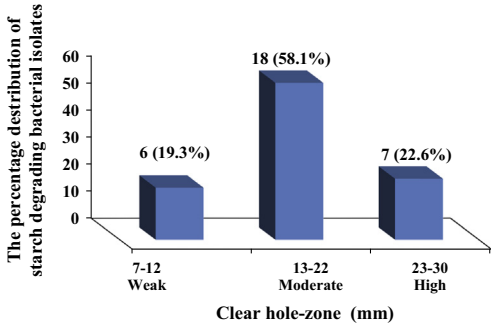

Fig. 1 The percentage distribution of starch degrading bacterial isolates into three categories that express as diameter of zone hydrolysis (mm).

All the previous isolates were tested for their capability to produce amylases on med.(2) by quantitative and qualitative assay at their proper temperature after 48 h. Data in Fig. 2(A) and (B) clearly show that α-amylase activity and SHR (starch hydrolysis ratio) ranged from 1.03 to 1.51 and 12.1 to 31.3 U ml^{-1} for mesophilic isolates at 30 °C, where it was 1.07–2.5 and 15–72.5 U ml^{-1} for thermophilic isolates at 50 °C, respectively. The statistical analysis (Analysis of variance and means of difference by Duncan[a,b]) of data in Fig. 2(A) and (B) proved that isolate E109 gave the highest potent being 72.5 U ml^{-1} at 50 °C, whereas isolate E20 gave 31.3 U ml^{-1} at 30 °C. From all previous data it could be stated that E109 was the pioneer since it gave 2.33-fold U ml^{-1} and 1.7-fold SHR comparing to the mesophilic isolate. Therefore isolate E109 was selected for further studies as a thermophilic productive isolate.

VaseeKaran et al. (2010) reported that the highest ratios of starch degradation ranged from 3.4 to 4 for tested isolates, while Alkando and Ibrahim (2011) indicated that the ratio of starch degradation by *B. licheniformis* was 1.5 compared to the other tested bacterial species.

Thermophilic microorganisms were capable of producing thermostable enzyme reported by Rasooli et al. (2008). These capabilities may be due to their molecular modifications at cellular and subcellular. Vaseekaran et al. (2010) and Panda et al. (2013) isolated three strains from soil receiving bakery waste produced the highest α-amylase activity at 90 °C. Jogezai et al. (2011) observed that amylases activity was maximal at 40 °C for *B. subtilis*.

Identification of the highest potent isolate

Phenotypic characteristics

The selected isolate was identified depending on its cultural, morphological and biochemical properties based on Bergey's Manual of systematic Bacteriology 2nd ed. (vol. 3: The

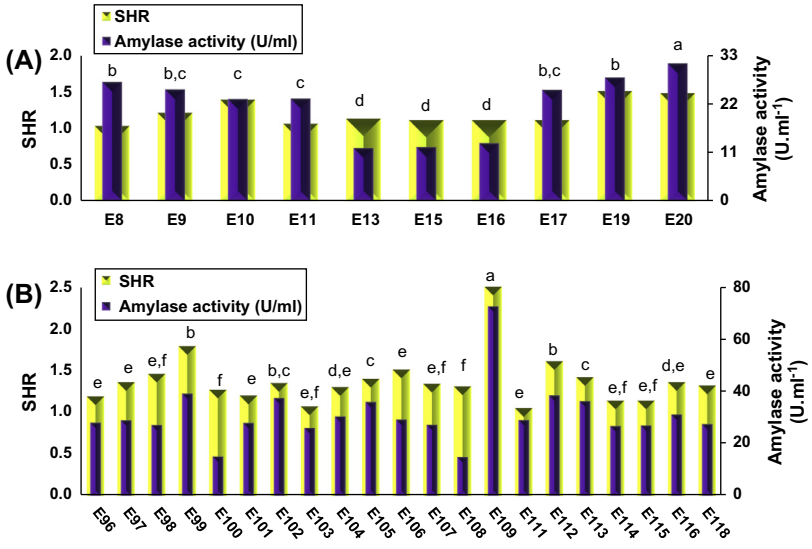

Fig. 2 Starch hydrolytic ratio and amylases activity U ml^{-1} obtained by different bacteria isolated from rhizosphere of Egyptian clover plants for 48 h as qualitative and quantitative estimations at (A) 30 °C, (B) 50 °C. Values in the same column (followed by letters within an alphabetic series) sharing the same letter do not differ significantly, according to Duncan (1955) at 5% level. Values followed by letters in different alphabetic series are significant, according to Duncan (1955) at 5% level.

Firmicutes) according to Niall and Paul (2009). The tested isolate E109 was Gram positive, rod shaped, motile, endo spore-forming bacterium, aerobic and positive for catalase, starch hydrolysis, casein hydrolysis, gelatin hydrolysis, citrate utilization, while gave negative results for nitrate reduction and Voges-Proskauer. These preliminary characteristics suggested that E109 was *B. amyloliquefaciens*.

Genotypic characteristics and the phylogenetic tree

Molecular identification and classification on the basis of 16S rDNA sequence analysis is important for correct identification of microbial species then morphological, physiological and biochemical characterization due to cumbersome and time-consuming (Poorani et al., 2009). Therefore, the most potent isolate (E109) with high potentiality for starch degradation was selected and confirmed identification using 16S rDNA sequence analysis. The genomic DNA of this isolate was amplified using PCR amplification of 16S rRNA gene. The results revealed efficient amplification; a single band of amplification DNA product 1500 bp was observed (Fig. 3).

The analysis of 16S rRNA gene of *B. amyloliquefaciens* (E109) was sequenced with R1 primer at the reverse direction and produced 1223 bp. The results of PCR sequences were compared with the other sequenced bacteria in National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) in Gene Bank and the Ribosomal Database Project (RDP) database showed similarity of derived sequences with some sequences belonging to the 16S small subunit rDNA of other bacteria.

Phylogenetic tree was conducted by taking the sequences obtained in blast search. Sequence obtained from BLASTN (nucleotide blast) was obtained in FASTA format and relation between each sequence could be known by multiple sequence alignment using a software CLUSTAL algorithm. The tree was generated using neighbor joining (NJ) a distance-based algorithm of phylogenetic analysis. Bacterial isolate (E109) was clustered. Based on 16S rRNA gene analysis, isolate E109 was grouped into genus *Bacillus*. The sequence of E109 was most closely related to *B. amyloliquefaciens* with similarity of 95% (Fig. 4).

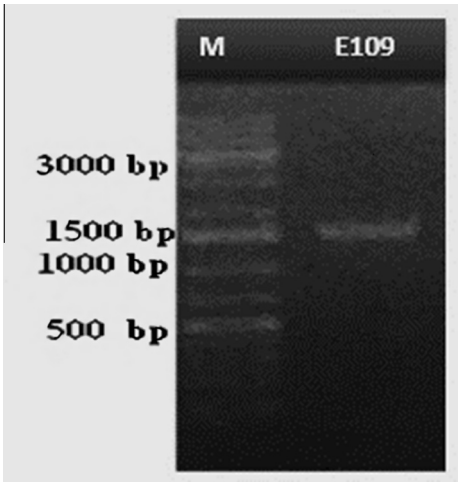

Fig. 3 PCR products for 16S gene with E109 bacterial isolate. M: 1 kbp DNA ladder.

Effect of starch substrates on the production of amylases by B. amyloliquefaciens at 50 °C by submerged fermentation

Biosynthesis of amylases was performed on agro-industrial wastes and by-products as starchy materials to solve pollution problems and obtain a low cost medium (Haq et al., 2005; Anto et al., 2006; Mukherjee et al., 2009). To investigate the effect of various agro-industrial residues as carbon sources such as corncobs, corn starch, potato starchy waste, rice bran, broken rice, rice husk, rice straw, wheat bran and wheat straw on growth and amylases activity produced by *B. amyloliquefaciens* at various time intervals, were incorporated with basal medium by replacing soluble starch (control 1%). Results from Fig. 5A clearly show that *B. amyloliquefaciens* grew exponentially during the first 36 h of fermentation periods on soluble starch, potato starchy waste, corn starch and broken rice, whereas increased to be 48 h on wheat bran, wheat straw, rice bran, rice husk, rice straw and corncobs.

Data presented in Table 1 and illustrated by Fig. 5A indicated that the highest amount of cell dry weight was recorded by *B. amyloliquefaciens* on potato starchy waste (0.72 gl^{-1}) followed by corn starch (0.62 gl^{-1}) after 36 h of fermentation periods. The lowest values of biomass were obtained in medium supplemented with wheat bran or corncobs, as compared with other tested carbon sources.

Data also indicated that the growth parameters (specific growth rate (μ), doubling time (t_d), multiplication rate (MR) and number of generations (N)) in log phase of growth curve of *B. amyloliquefaciens* were calculated on base med.2 supplemented with different carbon sources. The specific growth rate (μ), doubling time (t_d), multiplication rate (MR) and number of generations (N) for all tested carbon sources ranged from 0.044–0.084 h^{-1}, 8.3–15.8 h, 0.06–0.12 and 2.0–2.8, respectively as shown in Fig. 5B. *B. amyloliquefaciens* gave the maximum values of μ, MR & N in medium supplemented with potato starchy waste (0.084 h^{-1}, 0.12 & 2.6) and broken rice (0.083 h^{-1}, 0.12 & 2.8). Also, the lowest doubling time was recorded on potato starchy waste (8.3 h) followed by broken rice (8.5 h).

The α, β and γ amylases activity for *B. amyloliquefaciens* increased gradually during the fermentation periods and reached to maximum peak after 30 & 30 and 36 h on medium supplemented with potato starchy waste being 98.4 & 0.52 and 1.6 U ml^{-1} followed by broken rice being 93.4 & 0.24 and 0.61 U ml^{-1}, respectively (Table 1). The activity of enzymes observed in medium supplemented with corncobs or wheat bran loss 67.7% or 65.5% of α amylase activity, whereas loss 82.7% or 98.1% of β amylase activity also 87.5% or 97.5% of γ amylase activity, the significance reduction of enzymes activity may be due to thickness of the fermentation medium for wheat bran leading to decrease culture aeration, which were essential for the growth and amylases activity (Satyanarayana et al., 2004) also, corncobs was not suitable carbon source, it could be due to their content of starch is very poor, to be insufficient for amylases activity (Moreira et al., 2004).

These results are in line with the finding of many researchers, who found that natural starches such as maize starch, potato starch and rice starch were the best carbon sources for α-amylase production (Shigechi et al., 2004; Yang and Liu, 2004; Kunamneni et al., 2005; Najafi and Deobagka, 2005). Kamm and Kamm (2004) also, stated that potato and rice starches were suitable substrates for amylases production by *Bacillus* sp. Kumarai et al. (2011) obtained similar results

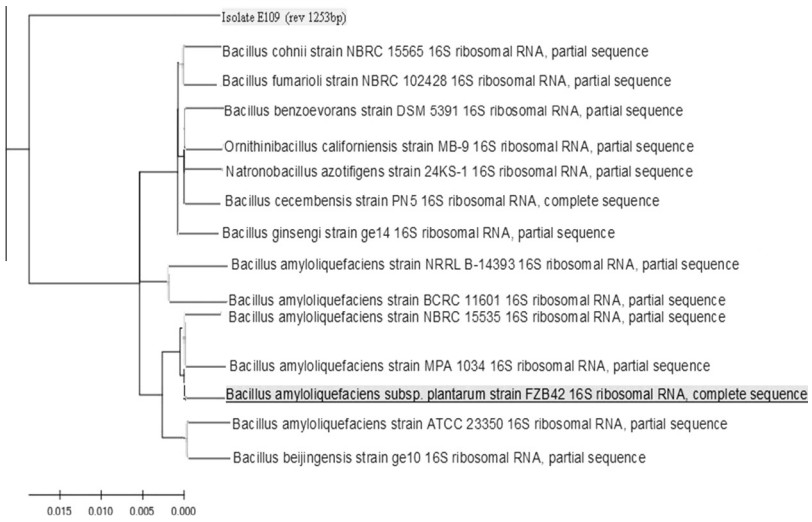

Fig. 4 Neighbor-joining tree based on 16S rRNA sequences of the genus *Bacillus* obtained from BLAST search showing the position of isolate and related strains.

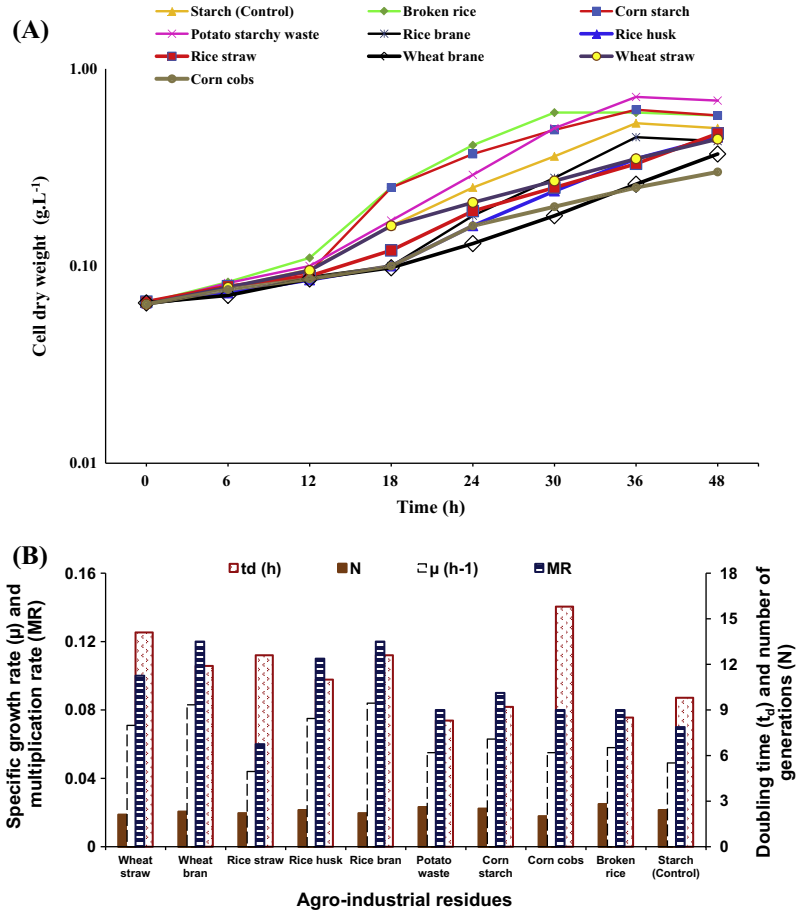

Fig. 5 Growth patterns of *B. amyloliquefaciens* as influenced by different carbon sources during 48 h at 50 °C using shake flasks as a batch culture. (A) Sigmoidal growth curve, (B) growth parameters.

which indicated that growth and amylases activity were high when maize starch was used as carbon source followed by potato starch.

Generally, it could be stated that the medium supplemented with potato starchy waste as the sole carbon source could be more favorable than soluble starch as control for α, β and γ

Table 1 Effect of different agro-industrial waste as starch substrates on growth and amylases activity of *B. amyloliquefaciens* on med.2 at 50 °C during 48 h using shake flasks as batch culture.

Starch substrates	Time (h)	CDW (gl^{-1})	Enzyme activity (U ml^{-1})			Starch substrates	Time (h)	CDW (gl^{-1})	Enzyme activity (U ml^{-1})		
			α	β	γ				α	β	γ
Soluble starch (control)	0	$0.07^{i,j}$	0.00^{m}	0.000^{j}	0.000^{l}	Rice bran	0	$0.07^{i,j}$	0.00^{m}	0.000^{j}	0.000^{l}
	6	$0.08^{i,j}$	$7.20^{k,l}$	0.021^{g}	0.009^{l}		6	$0.07^{i,j}$	$3.25^{l,m}$	0.002^{j}	0.003^{l}
	12	$0.10^{i,j}$	$12.9^{j,k}$	0.051^{f}	$0.015^{k,l}$		12	$0.09^{i,j}$	$7.63^{k,l}$	0.002^{j}	0.003^{l}
	18	$0.16^{h,i}$	$24.9^{g,h}$	0.075^{e}	$0.050^{h,i}$		18	$0.10^{i,j}$	$12.3^{j,k}$	0.003^{j}	0.006^{l}
	24	$0.25^{g,h}$	$36.4^{f,g}$	0.098^{d}	0.088^{g}		24	$0.13^{i,j}$	$19.1^{i,j}$	0.005^{j}	$0.011^{k,l}$
	30	$0.36^{e,f}$	$52.1^{d,e}$	0.130^{d}	0.131^{e}		30	$0.18^{h,i}$	$25.1^{g,h}$	$0.008^{i,j}$	$0.022^{k,l}$
	36	$0.53^{b,c}$	78.2^{c}	0.126^{d}	0.200^{d}		36	$0.26^{g,h}$	$33.9^{f,g}$	$0.010^{i,j}$	$0.040^{j,k}$
	48	$0.50^{b,c}$	$72.3^{c,d}$	0.116^{d}	$0.176^{d,e}$		48	$0.37^{e,f}$	$23.1^{g,h}$	$0.009^{i,j}$	$0.034^{j,k}$
Broken rice	0	$0.07^{i,j}$	0.00^{m}	0.000^{j}	0.000^{l}	Rice husk	0	$0.07^{i,j}$	0.00^{m}	0.000^{j}	0.000^{l}
	6	$0.08^{i,j}$	$15.0^{j,k}$	0.010^{h}	0.070^{g}		6	$0.07^{i,j}$	$3.80^{l,m}$	0.004^{j}	0.005^{l}
	12	$0.11^{i,j}$	$23.7^{g,h}$	0.030^{g}	0.150^{e}		12	$0.09^{i,j}$	$8.20^{k,l}$	$0.007^{i,j}$	0.007^{l}
	18	$0.25^{g,h}$	$36.8^{f,g}$	0.050^{f}	0.220^{d}		18	$0.10^{i,j}$	$17.9^{i,j}$	$0.010^{i,j}$	$0.012^{k,l}$
	24	$0.41^{d,e}$	$50.1^{d,e}$	0.150^{d}	0.350^{d}		24	$0.16^{h,i}$	$27.8^{g,h}$	$0.016^{i,j}$	$0.029^{k,l}$
	30	$0.55^{b,c}$	93.4^{b}	0.240^{c}	$0.480^{b,c}$		30	$0.24^{g,h}$	$42.2^{f,g}$	0.020^{g}	$0.039^{j,k}$
	36	0.60^{c}	88.1^{b}	0.230^{c}	$0.610^{b,c}$		36	$0.35^{e,f}$	$66.2^{c,d}$	0.019^{h}	$0.060^{h,i}$
	48	$0.58^{b,c}$	$79.7^{b,c}$	0.230^{c}	$0.570^{b,c}$		48	$0.45^{c,d}$	$54.4^{d,e}$	0.017^{h}	$0.052^{h,i}$
Corncobs	0	0.06^{j}	0.00^{m}	0.000^{j}	0.000^{l}	Rice straw	0	$0.07^{i,j}$	0.00^{m}	0.000^{j}	0.000^{l}
	6	$0.08^{i,j}$	$3.10^{l,m}$	$0.006^{i,j}$	$0.010^{k,l}$		6	$0.08^{i,j}$	$5.60^{k,l}$	0.004^{j}	$0.019^{k,l}$
	12	$0.09^{i,j}$	$6.19^{k,l}$	0.011^{h}	$0.019^{j,k}$		12	$0.09^{i,j}$	$10.8^{j,k}$	$0.006^{i,j}$	$0.031^{j,k}$
	18	$0.10^{i,j}$	$11.3^{j,k}$	0.020^{g}	$0.053^{h,i}$		18	$0.12^{i,j}$	$17.3^{i,j}$	0.013^{h}	$0.056^{h,i}$
	24	$0.16^{h,i}$	$17.9^{i,j}$	0.039^{g}	$0.091^{f,g}$		24	$0.19^{h,i}$	$27.4^{g,h}$	0.022^{g}	$0.072^{g,h}$
	30	$0.20^{h,i}$	$23.8^{g,h}$	0.064^{f}	0.131^{e}		30	$0.25^{g,h}$	$47.1^{d,e}$	0.032^{g}	$0.095^{f,g}$
	36	$0.25^{g,h}$	$31.8^{g,h}$	0.090^{e}	0.200^{d}		36	$0.33^{e,f}$	70.6^{c}	0.050^{f}	0.140^{e}
	48	$0.30^{g,h}$	$20.8^{i,j}$	0.082^{e}	0.182^{d}		48	$0.47^{c,d}$	$60.4^{d,e}$	0.045^{f}	0.128^{e}
Corn starch	0	$0.07^{i,j}$	0.00^{m}	0.000^{j}	0.000^{l}	Wheat bran	0	$0.07^{i,j}$	0.00^{m}	0.000^{j}	0.000^{l}
	6	$0.08^{i,j}$	$4.80^{k,l}$	0.010^{h}	$0.040^{j,k}$		6	$0.07^{i,j}$	$3.25^{l,m}$	0.002^{j}	0.003^{l}
	12	$0.09^{i,j}$	$11.0^{j,k}$	0.020^{g}	$0.050^{h,i}$		12	$0.09^{i,j}$	$7.63^{k,l}$	0.002^{j}	0.003^{l}
	18	$0.25^{g,h}$	$29.2^{g,h}$	0.040^{g}	$0.070^{g,h}$		18	$0.10^{i,j}$	$12.3^{j,k}$	0.003^{j}	$0.006^{k,l}$
	24	$0.37^{e,f}$	$50.1^{d,e}$	0.060^{e}	0.130^{e}		24	$0.13^{i,j}$	$19.1^{i,j}$	$0.005^{i,j}$	$0.011^{k,l}$
	30	$0.49^{c,d}$	89.5^{b}	0.110^{c}	$0.170^{d,e}$		30	$0.18^{h,i}$	$25.1^{g,h}$	$0.008^{i,j}$	$0.022^{j,k}$
	36	0.62^{c}	$80.2^{b,c}$	0.100^{c}	0.250^{d}		36	$0.26^{g,h}$	$33.9^{f,g}$	0.010^{h}	$0.040^{j,k}$
	48	$0.58^{b,c}$	71.2^{d}	0.090^{e}	$0.220^{d,e}$		48	$0.37^{e,f}$	$23.1^{g,h}$	0.009^{h}	$0.034^{j,k}$
Potato starchy waste	0	0.06^{j}	0.00^{m}	0.000^{j}	0.000^{l}	Wheat straw	0	0.06^{j}	0.00^{m}	0.000^{j}	0.000^{l}
	6	$0.08^{i,j}$	$11.6^{j,k}$	0.040^{gg}	$0.096^{f,g}$		6	$0.08^{i,j}$	$4.19^{k,l}$	0.002^{j}	0.003^{l}
	12	$0.10^{i,j}$	$25.8^{g,h}$	0.080^{e}	0.363^{d}		12	$0.10^{i,j}$	$14.6^{j,k}$	0.005^{j}	$0.006^{k,l}$
	18	$0.17^{h,i}$	$40.6^{f,g}$	0.140^{d}	0.523^{c}		18	$0.16^{h,i}$	$26.6^{g,h}$	$0.007^{i,}$	$0.015^{j,k}$
	24	$0.29^{g,h}$	$66.3^{c,d}$	0.305^{b}	0.881^{b}		24	$0.21^{h,i}$	$35.6^{f,g}$	0.010^{h}	$0.042^{j,k}$
	30	$0.50^{c,d}$	98.4^{a}	0.520^{a}	$1.265^{a,b}$		30	$0.27^{g,h}$	$51.3^{d,e}$	0.022^{g}	$0.065^{h,i}$
	36	0.72^{a}	94.4^{b}	0.505^{a}	1.600^{a}		36	$0.35^{e,f}$	70.3^{c}	0.040^{f}	$0.090^{f,d}$
	48	$0.69^{a,b}$	$80.1^{b,c}$	0.484^{a}	1.452^{a}		48	$0.44^{c,d}$	61.7^{d}	0.032^{g}	$0.080^{f,d}$

CDW = cell dry weight.
Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan (1955) at 5% level.

amylases activity, as it increased the enzymes produced by *B. amyloliquefaciens* about 1.26 & 4 and 8-fold, respectively.

Different potato starchy waste concentrations

Five concentrations of potato starchy waste ranging between 0.5% and 2.5% were used for amylases activity by the tested strain. Data presented in Fig. 6(A) and (B) clearly show that *B. amyloliquefaciens* grew exponentially during 36 h in medium containing potato starchy waste concentrations ranged from 0.5% to 2.5%. The highest figure of growth (0.84 gl^{-1} of cell dry weight) was recorded at 2% potato starchy waste after 36 h of fermentation periods, which increased about 16.6%, as compared with that produced at 1% potato starchy waste (control). The highest figures of specific growth rate (μ), multiplication rate (MR) and number of generations (N) were 0.092 h^{-1}, 0.13 and 3. The lowest doubling time (t_d) was achieved after 8 h.

Also, data presented in Table 2 clearly show that there was a gradual increase in activity of amylases by the tested strain with increase of potato starchy waste concentrations from 1.5% to 2.5% reaching a maximum growth and amylases activity at 2%. This treatment resulted to the highest values of α, β and γ amylases activity after 30, 30 & 36 h of fermentation periods being 155.2, 1.0 & 2.4 U ml^{-1}, respectively.

Statistical analysis revealed a high positive correlation coefficient (r^2) between potato starchy waste concentrations and

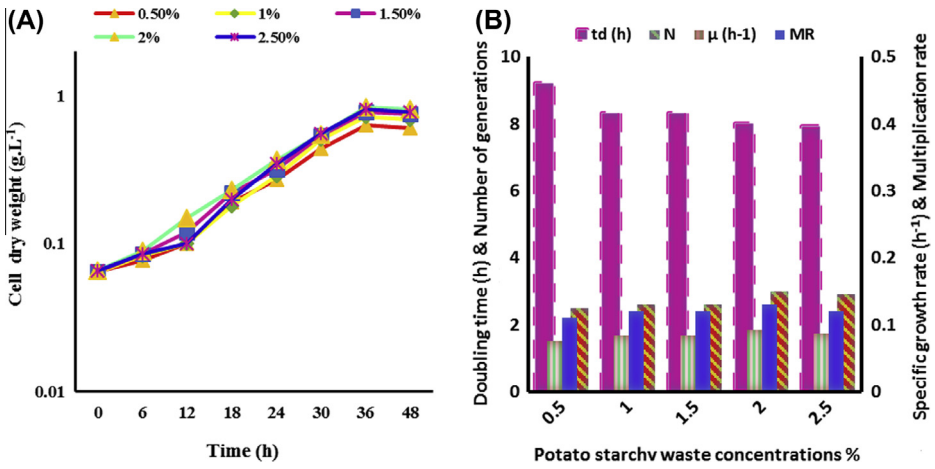

Fig. 6 Growth patterns of *B. amyloliquefaciens* as influenced by different potato starchy waste concentrations during 48 h at 50 °C using shake flasks as a batch culture. (A) Sigmoidal growth curve, (B) growth parameters.

Table 2 Effect of different potato starchy waste concentrations on growth and amylases activity of *B. amyloliquefaciens* on med.2 incubated at 50 °C during 48 h using shake flasks as a batch culture.

PSW conc.(%)	Time (h)	C.D.W (gl^{-1})	Enzyme activity (U ml^{-1})			PSW conc. (%)	Time (h)	C.D.W (gl^{-1})	Enzyme activity (U ml^{-1})		
			α	β	γ				α	β	γ
0.5	0	0.07^{g}	0.00^{n}	0.000^{i}	0.000^{h}	1.5	24	0.32^{d}	$75.80^{e,f}$	0.526^{d}	0.970^{d}
	6	0.08^{g}	5.21^{n}	0.048^{i}	$0.079^{g,h}$		30	0.54^{c}	$133.8^{b,c}$	0.750^{c}	1.206^{c}
	12	$0.10^{f,g}$	$15.8^{m,n}$	$0.094^{h,i}$	0.217^{g}		36	0.78^{a}	125.9^{c}	0.739^{c}	1.800^{b}
	18	0.19^{e}	$28.6^{l,m}$	0.118^{g}	$0.333^{f,g}$		48	0.76^{a}	120.9^{c}	0.678^{c}	1.765^{b}
	24	0.27^{e}	$47.6^{i,j}$	0.257^{f}	$0.468^{e,f}$						
	30	0.44^{d}	$77.6^{e,f}$	0.410^{e}	0.784^{e}	2.0	0	0.07^{g}	0.000^{n}	0.000^{i}	0.000^{h}
	36	0.63^{b}	$72.6^{f,g}$	0.373^{e}	1.000^{d}		6	$0.09^{f,g}$	$20.70^{m,n}$	$0.098^{h,i}$	0.251^{g}
	48	0.61^{b}	$60.6^{h,i}$	0.355^{e}	0.930^{d}		12	$0.15^{f,g}$	$43.50^{j,k}$	0.178^{g}	$0.505^{e,f}$
							18	0.23^{e}	$71.80^{f,g}$	0.327^{e}	0.815^{d}
1.0	0	0.06^{g}	0.00^{n}	0.000^{i}	0.000^{h}		24	0.37^{d}	98.30^{d}	0.696^{c}	$1.304^{b,c}$
	6	0.08^{g}	$11.7^{m,n}$	0.040^{i}	$0.096^{g,h}$		30	0.55^{c}	155.2^{a}	1.000^{a}	1.749^{b}
	12	$0.10^{f,g}$	$25.8^{l,m}$	$0.079^{h,i}$	0.363^{g}		36	0.84^{a}	149.2^{b}	0.969^{a}	2.400^{a}
	18	0.18^{e}	$40.5^{j,k}$	0.140^{g}	$0.523^{e,f}$		48	0.81^{a}	138.2^{b}	0.949^{a}	2.135^{a}
	24	0.29^{e}	$66.5^{g,h}$	0.305^{e}	0.881^{d}						
	30	0.51^{c}	98.4^{d}	0.520^{d}	1.265^{c}	2.5	0	0.07^{g}	0.000^{n}	0.000^{i}	0.000^{h}
	36	0.72^{b}	94.1^{d}	0.505^{d}	1.600^{b}		6	$0.09^{f,g}$	$17.10^{m,n}$	0.124^{g}	$0.178^{g,h}$
	48	0.69^{b}	$80.2^{d,e}$	0.484^{d}	$1.452^{b,c}$		12	$0.10^{f,g}$	38.20^{k}	0.224^{f}	0.284^{g}
							18	0.20^{e}	$65.70^{g,h}$	0.355^{e}	$0.471^{e,f}$
1.5	0	0.07^{g}	0.000^{n}	0.000^{i}	0.000^{h}		24	0.35^{d}	$88.98^{d,e}$	0.529^{d}	$0.706^{d,e}$
	6	$0.09^{f,g}$	$15.50^{m,n}$	0.149^{g}	0.268^{g}		30	0.56^{c}	149.0^{b}	0.900^{b}	1.252^{c}
	12	$0.12^{f,g}$	$30.20^{l,m}$	0.249^{f}	$0.459^{e,f}$		36	0.81^{a}	140.9^{b}	0.842^{b}	2.100^{a}
	18	0.22^{e}	$45.70^{i,j}$	0.348^{e}	0.616^{e}		48	0.78^{a}	129.9^{c}	0.773^{c}	1.716^{b}

PSW = potato starchy waste, CDW = cell dry weight.
Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan (1955) at 5% level.

each of cell dry weight, enzyme activity (α, β and γ amylases). r^2 values ranged from 0.87 to 0.95. Moreover, significant effect on bacterial growth and amylases synthesis by the tested strain was observed at all potato starchy waste concentrations. In similar studies, Mishra and Behera (2008) and Kanimozhi et al. (2014) noticed that the highest enzyme activity by *Bacillus* sp. was attained at 2.0% starch, whereas the maximum activity of enzyme by *Pseudomonas fluorescence* was ranged from 1.5% to 2.5% starch and decreased at 3.5% starch which have been reported by Karnwal and Nigam (2013). So, it could be stated that 2% potato starchy waste was the best concentration for the growth and enzymes activity by *B. amyloliquefaciens* resulting in 1.98, 7.69 and 12-fold for α, β and γ amylase activity, respectively as compared to control (1%).

Conclusion

Several bacterial isolates were isolated from Egyptian soil and were capable to grow and produce amylases. Among these isolates, E109 was found to produce the highest amylases activity on productive medium supplemented with 1% starch at 50 °C for 48 h using shake flasks as a batch culture. This isolate was

identified as *B. amyloliquefaciens* according to phenotypic tests and was confirmed by 16S rRNA gene sequencing. The present work has been taken up with a view of exploring the possibilities of using agro-industrial residues as a starch substrate for the production of amylases by the tested strain in submerged fermentation, which can hydrolyze starch to glucose. The use of inexpensive substrates can economize the process of production. This strain was capable to produce the highest α and β amylases after 30 h as well as γ amylase after 36 h in the medium containing 2% potato starch waste, respectively incubated at 50 °C using shake flasks (150 rpm) as a batch culture technique.

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