Optimization and purification of extracellular amylase from soil- bacteria against clinical biofilm-forming bacteria

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Background. Bacterial biofilms have become a significant and growing threat to human life, nature, and environmental health. This study aims to isolate amylase-producing bacteria from the terrestrial environment (soil) for investigating their general inhibition on some pathogenic human bacterial biofilm. **Methods**. A total of 75 amylase producing isolates were obtained by serial dilution and streaking method. Amylotic activity of these isolates was screened by a starch agar plate method. Isolates were characterized by morphological and biochemical methods. The optimum conditions for amylase production were monitored. Antagonistic activity of these isolates and purified amylase against multidrug-resistant pathogenic human bacteria by agar disk diffusion method. The sensitivity level of some standard antibiotics served as control. Finally, the antibiofilm efficacy of isolates filtrate and purified enzyme also monitored by spectrophotometric methods. **Results.** The isolated *Bacillus* species were *B. megaterium* (26.7%), *B. subtilis* (16%), B. cereus (13.3%), *B. thuringiesis* (10.7%), *B. lentus* (10.7%), *B. mycoides* (5.3%), *B. alvei* (5.3%), *B. polymyxa* (4%), *B. circulans* (4%) and *Micrococcus roseus* (4%). Interestingly, it was found that all isolates exhibited great antagonistic activity against the target pathogens. The greatest activity recoded by *B. alevi* (48 mm) against Staphylococcus aureus (MRSA) and the lowest activity recorded by *B. polymyxa* (12 mm) against *E. coli* while low inhibition recorded for amylase. The results revealed highly significant inhibition with percentages of 93.6 and 78.8% respectively. So, they represent a good tool for biofilm control in clinical and environmental applications.

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Abstract

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Methods. A total of 75 amylase producing isolates were obtained by serial dilution and streaking method. Amylotic activity of these isolates was screened by a starch agar plate method. Isolates were characterized by morphological and biochemical methods. The optimum conditions for amylase production were monitored. Antagonistic activity of these isolates and purified amylase against multidrug-resistant pathogenic human bacteria by agar disk diffusion method. The sensitivity level of some standard antibiotics served as control. Finally, the antibiofilm efficacy of isolates filtrate and purified enzyme also monitored by spectrophotometric methods.

Results. The isolated *Bacillus* species were *B. megaterium* (26.7%), *B. subtilis* (16%), B. cereus (13.3%), *B. thuringiesis* (10.7%), *B. lentus* (10.7%), *B. mycoides* (5.3%), *B. alvei* (5.3%), *B. polymyxa* (4%), *B. circulans* (4%) and *Micrococcus roseus* (4%). Interestingly, it was found that all isolates exhibited great antagonistic activity against the target pathogens. The greatest activity recoded by *B. alevi* (48 mm) against Staphylococcus aureus (MRSA) and the lowest activity recorded by *B. polymyxa* (12 mm) against *E. coli* while low inhibition recorded for amylase. The results revealed highly significant inhibition with percentages of 93.6 and 78.8% respectively. So, they represent a good tool for biofilm control in clinical and environmental applications.

Keywords: Amylase, *Bacillus*, Soil bacteria, Antibiofilm, Pathogenic bacteria.

Introduction

Bacterial biofilms have become a significant and growing threat to human life, nature, and environmental health (Hall-Stoodley et al. 2004). This is due to its association with a variety of infectious diseases also, they are infectious and can result in nosocomial infections (Jamal et al. 2015). Biofilms also showing high resistance to different antibiotics (Sharma et al. 2019). The first step in biofilm formation is the initial attachment to surfaces (Costerton et al. 1999). In a study by Bryers (1988), he was found that extracellular polymers and gas production of bacteria

involved in sloughing biofilm from surfaces. Using enzymes also is a good strategy for biofilm removal, as enzymes are rabidly biodegradable and harmless to the environment (Xavier et al. 2005). Amylase is one member of digestive enzymes that hydrolyzes glycosidic bonds of starch to form maltotriose glucose, dextrin, and maltose so, it termed as glycosidic hydrolases (Kaur et al. 2012). Production of amylase by microbes is more advantageous due to the high production rate and can easily engineer into desired products (lonsane and Ramesh, 1990). Soil is the main part of the terrestrial environment that occupied with a large association of microorganisms compared with aquatic environments. This is due to its higher content of organic and inorganic materials (Murphy et al. 2007). Among terrestrial bacteria, *Bacillus* sp. that is a good choice of the source as amylase producers such as *Bacillus subtilis*, *Bacillus cereus* and *Bacillus polymyxa* (El-Fallal et al. 2012; Dash et al. 2015). Amylase from *Bacillus* is thermostable, has retention to extreme pH, osmolarity and high pressure and this is important in industrial production (Islam et al. 2017). Antibiotics such as bacitracin, gramicidin S, polymyxin and tyrotricidin produced by *Bacillus* sp. exhibited great efficacy against Gram-positive and Gram-negative bacteria (Perez et al. 1992; 1993; Yilmaz et al. 2006). In this study, *Bacillus* sp. was isolated from soil, identified by morphological and biochemical assays. Antimicrobial activity of these isolates was investigated against five human pathogenic strains. Purification of amylase was done after its optimization and studying the best condition of temperature, pH, incubation period and starch concentration that gives the highest activity for purification. Finally, the antibiofilm activity of the isolates filtrate and the purified amylase from these isolates were monitored.

Materials & Methods

1- Soil samples collection. One hundred soil samples were collected from different places during November 2019 from the Luxor governorate, Egypt. Samples were collected in sterile plastic bags under aseptic conditions and transported to the laboratory (Reed and Rigney 1947).

One gram of soli added to 5 ml tryptic soy broth (Oxoid) modified with 1% starch as enrichment broth. Samples incubated at 37^{o}C for 24h.

2- Screening and isolation of amylase-producing bacteria. Serial dilution techniques are one of the most precise methods for bacterial isolation from soil (Jamil et al. 2007; Rasooli et al. 2008). Serial dilutions were done up to 10^{-7}. 100 µl from each dilution was transferred aseptically and spread into tryptic soy agar media fortified with 1% starch. The plates were incubated at 37^{o}C for 24h and CFU/ml was determined. The plates were then flooded with iodine that turns blue when reacting with starch that not hydrolyzed. If starch is hydrolyzed, a clear halo zone was observed around colonies that produce amylase against a dark blue background (Abd-Elhahlem et al. 2015; Gupta et al. 2003). Bacterial isolates were further subcultured to obtain a pure culture. Isolates were identified by standard morphological techniques based on colony shape, Gram's staining, spore formation and biochemical characterization (Koneman et al. 1992; Collins and Lyne 1984; Cruickshank et al. 1975). Isolates were then maintained in 70% sterilized glycerol stock under -70 ^{o}C for further use.

3- Selection of isolates for amylase purification. The selection of isolates for extraction and purification of amylase, for studying the antibiofilm activity of the purified amylase against some human pathogenic bacteria, occurred according to starch hydrolysis ratio (SHR) that calculated from the following equation (Pranay et al. 2019).

SHR= clear halo zone diameter (mm) / colony growth diameter (mm).

Where isolates were subcultured on starch agar plates. Then the plates were incubated for 24h at 37^{o}C. After incubation, the plates flooded with iodine. Finally, SHR was calculated according to the above equation.

4- Optimization of amylase production.

a- Effect of temperature and incubation periods. The starch nutrient medium was prepared and the pH adjusted to 7.5. The medium inoculated with the tested isolates. The cultured allowed to grow on a rotatory shaker (250 revs/min) at different temperatures ranged from 25 to 55^{o}C for 48h. Then, 20 ml of each culture for all temperatures at different time intervals (18, 24 and 48h) was taken and centrifuged to remove the bacterial cells. Finally, the supernatant was collected for assaying amylase activity (Nimisha et al. 2019).

b- Effect of pH. The starch nutrient medium was prepared and pH was adjusted to different values from 5 to 10. Each isolate was inoculated into a set of this medium and grown at 50^{o}C for 24h. Then, 20 ml of each were collected and treated as above for determination amylase activity (Nimisha et al. 2019).

c- Effect of starch concentration. *Bacillus* isolates were grown on nutrient broth medium at pH 9 except *Bacillus subtilis* at grown at pH 7. Fresh medium was prepared with the addition of different soluble starch quantities to give a final concentration of 0.1, 0.5, 1, 1.5, 2, 2.5 and 3%. Then isolates were inoculated each in a set of this medium and grown at 50^{o}C for 24h. Finally, amylase activity was determined (Nimisha et al. 2019).

5- Determination of amylase activity under optimum conditions. The assay mixture containing 2 ml of a solution of 1% starch in 50 mM sodium phosphate buffer (pH7) and 0.1 ml enzyme solution. After 10 min. of incubation at 40^{o}C, the reaction was stopped by adding 2 ml of 3,5 dinitrosalicylic acid (DNS) reagent and the tubes were heated at 100^{o}C for 5 minutes. The absorbance was measured spectrophotometrically at 540 nm against a blank containing buffer instead of the culture supernatant. The amount of reducing sugars was calculated from a standard curve constructed by using maltose (Meyer et al. 1951). Protein was determined using the method of Bradford (1976).

6- Enzyme purification.

a- Ammonium sulfate precipitation. The crude amylase enzyme was brought to 45% saturation with ammonium sulfate and kept overnight in a cold room at 4^{o}C. The precipitate was discarded, while the supernatant was brought to 85% saturation with ammonium sulfate and centrifuged at 8000 rpm at 4^{o}C for 10 min. The precipitate from this step was collected and stored at 4^{o}C (Shinde & Soni 2014).

b- Dialysis. This step was conducted to exclude the remains of ammonium sulfate and concentrate the enzyme. The dialysis tubes, which were previously soaked in 0.1 M phosphate buffer, pH 6.2 were used for dialysis of the precipitate. The precipitate was dissolved in 0.1 M phosphate buffer and dialyzed against the same buffer (Roe 2001).

c- DEAE sephadex A-25. The crude enzyme preparations of the six culture filtrate were applied separately to a column of DEAE-Sephadex A-25. The enzyme was eluted with a linear gradient of sodium chloride (0 – 0.4 M) in 200 ml of sodium phosphate buffer (0.05 M and pH 7), the flow rate was adjusted to 1 ml per 1 min. and the 200 ml of eluents were collected into 40 tubes (1x7 cm) using an automatic circular fraction collector. Enzyme activity and protein concentration were determined in each fraction as described in the assay method. Fractions of the highest specific activity were pooled together and kept for further studies.

7- Antibacterial activities.

7.1. Antagonistic efficacy of the isolated bacteria. The antagonistic efficacy of all isolates was studied against 5 human pathogenic strains. The used pathogenic strains in the study work (*Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (MRSA) were kindly obtained from the International Luxor hospital in Luxor province, Egypt. The screening was done by disc diffusion method. All bacteria were

149 cultured on TSB modified with 1% starch and incubated at 37°C at 24h. The isolated bacterial
150 cultures were centrifuged to exclude the cell debris (6000 rm – 15 min. - Biofuge). Then 20 ml of
151 TSA modified with 1% starch was poured in a sterile Petri plate (100 mm diameter). 100 µl of 5
152 tested pathogens were streaked on the plates and by using sterile borer, wells of 6 mm were
153 punched in the plates. The wells were then filled with 100 µl of the isolated bacteria filtrate. The
154 plates were then incubated at 37°C for 24h. The inhibition zone was measured using a ruler in
155 mm (Reinheimer et al., 1990). Standard antibiotics were served as control using Kirby Bauer
156 disk diffusion method (Bauer et al., 1966). The used antibiotics were Chloramphenicol (C; 30
157 µg, Oxoid), Oxacillin (OX; 1 mcg, Bioanalyse®), Vancomycin (VA; 30 mcg, Bioanalyse®),
158 Ampicillin/Sulbactam (SAM; 10/10 mcg, Bioanalyse®), Penicillin G (P; 10 U; Bioanalyse®),
159 Erythromycin (E; 15 mcg, Bioanalyse®), Sulfamethoxazole/Trimethoprim (SXT; 23.75/1.25 µg,
160 BBL™), Cefotaxime (CTX; 30 mcg, Bioanalyse®), Gentamycin (GM; 10 µg, Bioanalyse®),
161 Meropenem (MEM; 10µg, Bioanalyse®), Piperacillin (PIP; 100 µg, Bioanalyse®) and
162 Piperacillin-tazobactam (PTZ; 100/10 µg, Bioanalyse®) . Interpretation of the results was done
163 according to Clinical Laboratory Standard Institute guidelines (CLSI 2017) to determine if the
164 tested pathogens were resistant, intermediate or sensitive against the used antibiotics.

165 **7.2. Antibacterial activity of purified amylase enzyme from the selected isolated *Bacillus*.**
166 100 µl of purified amylase from the selected isolates according to SHR were put in the wells of
167 the agar plates inoculated with the target strains as described earlier. The plates then incubated at
168 37°C for 24h. The diameter of the hallo zone was measured using a ruler expressed in mm.

169 **7.3. Biofilm formation assay.** The ability of the tested pathogens: *E. coli*, *Klebsiella*
170 *pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeuroginosa*, and *Staphylococcus aureus*
171 (MRSA) for biofilm formation was determined using 96-well polystyrene plates (Seper et al.

2011) after modifications by Salem et al. (2015) as follows: isolates were subcultured on tryptic soy agar for 24h at 37^{o}C, suspended in tryptic soy broth and adjusted to an OD_{595} of 0.02. 130 µl of each adjusted isolate culture were put in the microtitre plate (U bottom, Sterilin) at 37^{o}C for 48h. After incubation the wells were washed with distilled water (6 times), Furthermore, the wells were stained with 0.1% crystal violet for 10 min. the wells were again washed with distilled water (4 times) to remove excess stain. Finally, the wells were destained by 210 µl of ethanol 96% and the OD_{595} was read using infinite® F50 Robotic (Ostrich) Microplate Plate to quantify the amount of biofilm.

7.4. Antibiofilm activity of the isolated *Bacillus sp*. filtrate and its purified amylase enzyme. The effect of the isolated bacteria filtrate and purified amylase of selected isolates as antibiofilm against five human biofilm former pathogenic bacteria were done by spectrophotometric methods as follows: a fresh culture of the isolates was prepared and adjusted to 0.5 McFarland (10^6 CFU/ml). 30 µl (This volume was selected according to preliminary experiment) of these cultures and purified amylase enzyme was added to130 µl the tested pathogens at OD_{595} of 0.02 after 24h incubation for allowing biofilm formation. The plates then incubated for 48h and then stained with crystal violet as described earlier. Wells without isolated cultures or amylase served as control.

Results

1- Screening and isolation of amylase producing bacteria. After streaking of diluted soil samples that collected from Luxor, Egypt, on starch agar plates and flooding the plates with an iodine solution to observe the halo zone around some colonies that indicating amylase production, it was found that a total of seventy-five bacterial isolates were amylase producer. Bacterial isolates were selected according to their amylolytic activity (Fig.1). Isolates were further characterized by morphological and biochemical tests as shown in Table (1 and 2). The

results showed that the seventy-five isolates were 19 *B*. *megaterium*, 12 *B*. *subtilis*, 10 *B*. *cereus*, 8 *B*. *thuringiesis*, 8 *B*. *lentus*, 4 *B*. *mycoides*, 4 *B*. *alvei*, 3 *B*. *polymyxa*, 3 *B*. *circulans* and 3 *Micrococcus roseus*. The highest prevalence recorded to *B*. *megaterium* (26.7%) and the lowest one was to *B*. *circulans* and *Micrococcus roseus* (4%). CFU of the amylase producing bacteria in one hundred soil samples ranged from $115x10^3$ – $198x10^5$ CFU/ml Table (1).

2- Optimization of amylase production. Starch hydrolysis rate as shown in (Table 2, Fig. 1d) lead to the selection of six isolates for amylase purification with the highest hydrolysis rate namely *B. alvei, B. thuringiesis, B. megaterium, B. subtilis, B. cerus,* and *B. lentus* with SHR of 6.0, 5.67, 5.33, 5.0, 4.0 and 3.5 mm respectively. The optimization was done to select the best conditions as temperature (25^{o}C), incubation period (24h), pH (8), and starch concentration (1.5-2%) that gives the highest amylase activity for using in purification (Fig. 2).

a- Effect of temperature and time intervals. All isolates showed maximum amylase production after 24h. *B. megaterium, B. subtilis*, and *B. cereus* showed maximum amylase production at 45^{o}C while, other isolates showed maximum amylase production ranged from 25-30^{o}C (Fig. 2 a and b).

b- Effect of pH. All *Bacillus* isolates give maximum amylase production at pH 8.0 except *B. subtilis* produced amylase maximally at pH 7.0 (Fig. 2c).

c- Effect of substrate concentration. The results showed that *B*. *subtilis* and *B*. *cereus* give maximum amylase production at 1.5 % of soluble starch. The remain isolates give maximum amylase production at 2.0 % of soluble starch (Fig. 2d).

d- Enzyme activity. As shown in Table (4), the highest amylase activity produced by *B*. *alvei* (96.02 U/ml) followed by *B*. *thuringiesis* (88.64 U/ml). While, *B*. *megaterium*, *B*. *subtilis* and *B*.

cereus produced amylase activity of 80.03, 76.0 and 55.9 U/ ml respectively. On the other hand, *B*. *lentus* produced the lowest amylase activity of 45.69 U/ml.

3- Antibacterial activity.

3.1. Antagonistic efficacy of the isolated bacteria and purified amylase enzyme from selected isolates. This study established the antimicrobial activity of the isolated amylase producing bacteria and purified amylase against five human pathogenic bacteria, the sensitivity level of some standard antibiotics served as a control, the results were shown in (Table 3; Fig. 1, a-b and c). It was found that *E*. *coli* was resistant to sulfamethoxazole-trimethoprim, gentamycin, cefotaxime, piperacillin, and piperacillin-tazobactam. While it was intermediate to ampicillin-sulbactam and sensitive to chloramphenicol and meropenem. Interestingly, it was found that all isolated bacteria have better antimicrobial activity against *E.coli* with the highest effect recorded to *B*. *polymyxa* (36 mm) and the lowest effect recorded to *B*. *subtilis* and *B*. *cereus* (12 mm). On the other hand, *B*. *mycoides* and *M. roseus* had no antimicrobial activity against *E. coli* (Fig.1c). *K. pneumoniae* was resistant to all tested antibiotics while it was intermediate to ampicillin sulbactam. It is worth mentioning that, all isolates had great antimicrobial effects with highest inhibition recorded to *B*. *megaterium* (26 mm) and the lowest effect recorded to *B*. *polymyxa* (17 mm) (Table 3). In contrast, *B*. *mycoides* and *M. roseus* were resistant to *K. pneumoniae* (Fig.1c). *A. baumanii* was resistant to all tested antibiotics and sensitive to only chloramphenicol. On the other hand, all isolated bacteria had better antibacterial effects against tested pathogens with the greatest effect was to *B*. *alvei* and *B*. *cirulans* (39 mm) and the lowest effect was to *B*. *subtilis* and *B*. *thuringiesis* (21 mm) (Table 3). Although, no inhibitory effect recorded to *B. mycoides* and *Micrococcus roseus* against the tested pathogens (Fig.1c). The susceptibility level of *P. aeruginosa* indicated that it was resistant to sulfamethoxazole-trimethoprim, cefotaxime,

gentamycin, meropenem and piperacillin. But it was intermediate to chloramphenicol and ampicillin-sulbactam, sensitive to piperacillin-tazobactam. It is important to say that all isolates had a great antibacterial effect against tested pathogens with the highest effect recorded to *B. lentus* and *B. cirulans* (32 mm) and the lowest on to *B. subtilis* (15 mm). Finally, *S. aureus* (MRSA) was resistant to oxacillin, vancomycin, penicillin G, cefotaxime and Gentamycin while, it was intermediate to chloramphenicol, erythromycin, and sulfamethoxazole-trimethoprim. On the other hand, the isolated amylase producing bacteria had a better antibacterial effect on tested pathogens with the greatest effect recorded to *B. alvei* (48 mm) and the lowest effect recorded to *B. cereus* (14 mm) (Table 3). but *B. mycoides* and *M. roseus* did not affect *S. aureus* (Fig.1c). Antibacterial activity of purified amylase from selected isolates showed very little effect on *E. coli* and *K. pneumoniae* (The highest inhibition diameter was 7.5 mm) but no effect recorded against the other tested pathogens (Table 3).

3.2. Antibiofilm activity of isolated bacterial filtrate and purified amylase enzyme from selected *Bacillus* isolates. The ability of pathogenic bacterial strains for biofilm formation by spectrophotometric methods was confirmed before the antibiofilm treatments with bacterial filtrate and purified amylase enzyme. As shown in Fig. (3 and 4), the antibiofilm activity was screened by a spectrophotometric method using crystal violet staining. Our results showed that *B. megaterium* inhibits biofilm of *E. coli*, *K. pneumoniae*, *A. baumanii*, *P. aeruginosa*, *Staphylococcus aureus* (MRSA) significantly with percentages of 63.3, 91.8, 73.2, 27.9 and 52.7% respectively (Fig. 3 T1). The second isolate was *B. subtilis*, this isolate also recorded high significant effect in biofilm inhibition with percentages of 74, 93, 73.7, 54 and 61.9% after 48h of treatment for *E. coli*, *K. pneumoniae*, *A. baumanii*, *P. aeruginosa* and *S. aureus* (MRSA), respectively (Fig. 3 T2). Antibiofilm activity of *B. cereus* showed significant inhibition

264 percentages of 64.5, 92.6, 61.1, 21.6 and 58.6% after 48h treatment of the tested pathogens
265 respectively (Fig. 3 T3). The efficacy of *B. thuringiesis* also has been monitored, the results
266 revealed high significant inhibition of biofilm with percentages of 39.6, 92, 74.3, 50.8 and 66.3%
267 respectively (Fig. 3 T4). *B. lentus* recorded high significant inhibition biofilm with percentages
268 of 66, 90.8, 74.5, 52.8 and 73.3% (Fig. 3 T5). *B. alvei* showed also highly significant inhibition
269 with percentage of 31.9, 68.7, 74, 56.3, 81.6% (Fig. 3 T6). *B. polymyxa* and *B. circulans* also
270 have great efficacy as antibiofilm against *E. coli, K. pneumoniae, A. baumanii, P. aeruginosa*
271 and *S. aureus* (MRSA) with the percentage of (68.9, 92.8, 72.6, 56.7, 70.9%) and (49, 93.7, 71.4,
272 28.2, 77.3 %) respectively after 48h treatment Fig. (3 T7 and T8). Efficacy of purified amylase
273 enzyme as antibiofilm also had been monitored against the same tested pathogens. Results
274 revealed that maximum activity was recorded by *B. alvei* (enzyme activity, 96.02 U/ ml) with
275 significant inhibition percentages of 74, 78.8, 78.2, 76.2 and 74.5%, respectively (Fig. 4 T6). *B.*
276 *thuringiesis* (enzyme activity, 88.64 U/ ml) comes after *B. alvei* as antibiofilm with percentages
277 of 69.7, 75, 74.1, 68 and 69.5% on *E. coli*, *K. pneumoniae*, *A. baumanii*, *P. aeuroginosa* and
278 *S.aureus* (MRSA), respectively (Fig. 4 T4). *B. megaterium* (enzyme activity, 80.03 U/ ml) and *B.*
279 *subtilis* (enzyme activity, 76.0 U/ml) comes after *B. alvei* and *B. thuringiesis* inefficacy of
280 biofilm inhibition with percentages of (65.6, 71, 71.6, 61.6, 64.3%) and (62.4, 67.8, 70, 50,
281 61.5%) respectively after 48h of treatment (Fig 4 T1 and T2). The lowest efficacy as antibiofilm
282 recorded to *B. cereus* (enzyme activity, 55.9 U/ml) and *B. lentus* (enzyme activity, 45.69 U/ml)
283 where after 48h treatment biofilm of the tested pathogens decrease by (55, 66.7, 68, 46.4 and 59
284 %) and (41.3, 60.6, 62, 32.4 and 53.7%) respectively Fig. (4 T3 and T5).

285 **Statistical analysis.** Data were analyzed using the Mann-Whitney U test or a Kruskal-Wallis
286 test followed by post hoc Dunn's multiple comparisons. Differences were considered significant
287 at P values of ≤ 0.05. For all statistical analyses, GraphPad Prism version 5 was used.
288
289 ## Discussion

290 Since pathogenic bacteria became extremely resistant to generally used antibiotics. Besides, the
291 unused antibiotics may dispose in the sewage system and if they are not depredated during
292 sewage treatment in the soil they may reach ground, surface and drinking water. This will be a
293 great fate and risk entering the environment (Kümmerer 2003). Searching for new natural
294 antimicrobial became important requirements for pharmaceutical industries to overcome the
295 problem of multidrug-resistant strains (Schmidt, 2004). Antibiotic production is a feature of
296 several soil organisms that represents a survival mechanism by which organisms can eliminate
297 competition (Talaro and Talaro 1996; Jensen and wright 1997). Genus *Bacillus* is among
298 terrestrial strains that can produce inhibitory compounds from peptide derivative and
299 lipopolypeptides antibiotics (Stein 2005; Tamehiro et al. 2002; Mannanov and Sattarova 2001).
300 Our study based on isolation of amylase producing- bacteria from soil and the results of isolation
301 indicated that *Bacillus* species were obtained by 96% from the total number of isolates (Table. 1,
302 2). Oscaiz et al. 1999; Yilmaz et al. 2006, found that isolated bacteriocin producing strains such
303 as *Bacillus* sp. were active against Gram-negative and Gram-positive bacteria. Perez et al. 1992
304 and Aslim et al. 2002, displayed that *B. subtilis*, *B. thuringiesis* and *B. megaterium* have
305 antibacterial activity against *E. coli* and *Pseudomonas aeuroginosa*. Our study confirmed the
306 antibacterial activity of the isolated *Bacillus* sp. (*B. megaterium*, *B. cereus*, *B. subtilis*, *B.*
307 *thuringiesis*, *B. lentus*, *B. alvei*, *B. polymyxa* and *B. circulans*) against multidrug-resistant human
308 pathogenic strains (*E. coli*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas*

309 *aeruginosa*, and *S. aureus* (MRSA) that confirmed by our results on standard antibiotics (Table.
310 3). The results revealed that 80% of the isolated species have an inhibitory effect against the
311 tested pathogens as shown in Table (3) Fig 1 (a and b). On the other hand, results revealed that
312 purified amylase enzyme obtained from these isolates has a very little antibacterial effect against
313 *E. coli* and *K. pneumoniae* while no effect verified against *A. baumanii*, *P. aeuroginosa* and *S.*
314 *aureus* (MRSA) (Table 3; Fig. 1c) in agreement with Kalpana et al. 2012 who confirmed that
315 amylase enzyme has no antibacterial effect. Bacterial biofilm is considered a great threat to
316 human life because of its antibiotic resistance (Costerton et al. 1999; Khan et al. 2014). Also, it
317 contributes to the chronicity of infections (Stewart and Costerton 2001). Our results revealed
318 highly significant inhibition of biofilm produced by *E. coli*, *K. pneumoniae*, *A. baumanii*, *P.*
319 *aeuroginosa* and *S. aureus* (MRSA) using some isolated *Bacillus* species extracellular
320 metabolites (filtrate) Fig. (3) . The results showed the greatest inhibition obtained by *B. circulans*
321 against *K. pneumoniae* biofilm with a percentage of 93.6% and the lowest inhibition recorded by
322 *B. cereus* against *P. aeruginosa* biofilm with a percentage of 21.7%. This may be due to presence
323 of some extracellular and intracellular metabolites in the medium where intracellular can release
324 into the extracellular medium when accumulated in the cell and this has been explained by the
325 metabolic overflow theory (Horak et al. 2019; Pinu et al. 2018; Pinu et al. 2017). *Bacillus* also
326 has great efficacy for the production of carbohydrate-active enzymes, bioactive compounds,
327 secretion of a variety of extracellular metabolites and lytic enzymes (Abdel-Aziz 2013). Where,
328 *Bacillus* species are the most popular producing peptide antibiotic compounds such as
329 polymyxin, colistin, and circulin (Katz and Domain 1997; Atanasova-Pancevska et al. 2016).
330 Antimicrobials are unable to penetrate biofilm due to the presence of extracellular polymeric
331 substances (EPS) that considered a primary matrix of biofilm (Flemming et al. 2007). It

produced by bacteria that bind with surfaces for some periods (Xavier et al. 2005). So, this layer plays an important role in facilitating bacterial attachment (Stoodley et al., 2002). Capture nutrients (Gomez-Suarez et al. 2002) also stabilizer of the biofilm structure and protecting the barrier of bacteria inside biofilm (Ploux et al. 2007). EPS is highly hydrated because it incorporated a large amount of water into its surface by hydrogen bonding (Prakash et al. 2003). So, the treatment of biofilm depends on the removal of EPS. Enzymes are playing an important role in removing and inactivation of bacterial biofilm (Johansen et al. 1997; Molobela et al. 2010; Lequette et al. 2010). So, significant reduction of biofilm for *E. coli*, *K. pneumoniae*, *A.baumanii*, *P. aeruginosa*, and *S. aureus* (MRSA) that revealed by our results due to using purified amylase enzyme of selected *Bacillus* isolates may be due to the above-mentioned explanations Fig. (4). The greatest inhibition recorded to amylase of *B. alvei* against *K. pneumoniae* with a percentage of 78.8% and the lowest effect was to amylase of *B. lentus* against *P. aeruginosa* with percentages of 20.6%. Results also indicated a great inhibition of biofilm obtained amylase enzyme of *B. alvei* followed by *B. thuringiesis*, *B. megaterium*, *B. subtilis*, *B. cereus* and *B. lentus*. This may be due to the increased enzyme activity of each of them. Where, enzyme activity was 96.02, 88.64, 80.03, 76.0, 55.9 and 45.69 U/ml respectively Table (4). It is worth mentioning that the filtrate of isolated bacteria showed great antibiofilm activity compared with the purified amylase enzyme from the selected isolates.

Conclusions

In the present study, for the first time, filtrate of different isolated *Bacillus sp*. (amylase producer) from the soil, was found to be an excellent antibiofilm agent against five human pathogenic biofilm former strains. The highest inhibition percentage was 93.6 %. Antibiofilm activity of purified amylase also monitored with inhibition percentages of 78.8%. Hence, our study was evidence that the filtrate of *Bacillus* was a good antibiofilm in clinical applications.

Further studies are in progress to identify the exact composition of the filtrate and its active compounds.

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Table 1(on next page)

Prevalence of *Bacillus* species isolated from soil.

[a]: The isolated amylase producing bacteria from soil. [b]: number of each isolated type from the total number of the positive isolated sample, [c:] percentage of each isolate, [d:] Average of colony-forming unit of amylase producing bacteria per ml of 100g soil samples (highest value- lowest value).

Table (1): Prevalence of *Bacillus* species isolated from soil

Isolates[a] / Parameters	No. of isolates[b]	Percentage[c] (%)	CFUml^{-1}[d]
Bacillus megaterium	20	26.7	$115\times10^3 - 198\times10^5$
Bacillus subtilis	12	16	
Bacillus cereus	10	13.3	
Bacillus thuringiesis	8	10.7	
Bacillus lentus	8	10.7	
Bacillus mycoides	4	5.3	
Bacillus alvei	4	5.3	
Bacillus polymyxa	3	4	
Bacillus circulans	3	4	
Micrococcus roseus	3	4	
Total 10	75	100	

[a]: The isolated amylase producing bacteria from soil. [b]: number of each isolated type from the total number of the positive isolated sample, [c:] percentage of each isolate, [d:] Average of colony-forming unit of amylase producing bacteria per ml of 100g soil samples (highest value- lowest value).

Table 2(on next page)

Biochemical activities of amylase- producing bacterial isolates and its starch hydrolysis rate.

Morphological and biochemical tests used for identification of the isolated bacteria. +:

Positive, -: Negative, [b]: starch hydrolysis rate.

Table (2): Biochemical activities of amylase- producing bacterial isolates and its starch hydrolysis rate.

Isolate / Tests[a]	Gram reaction	Motility	Catalase	Egg yolk lecithinase	Nitrate reduction	Vogas proskauer	Citrate utilization	Gelatin hydrolysis	Starch hydrolysis	Indole production
Bacillus megaterium	+	+	+	-	-	-	+	+	+	-
Bacillus subtilis	+	+	+	-	+	+	+	+	+	-
Bacillus cereus	+	+	+	+	+	+	+	-	+	-
Bacillus thuringiesis	+	+	+	+	-	+	+	-	+	-
Bacillus lentus	+	+	+	-	-	-	-	+	+	-
Bacillus mycoides	+	-	+	+	-	+	+	-	+	+
Bacillus alvei	+	+	+	-	-	+	-	+	+	+
Bacillus polymyxa	+	+	+	-	+	+	-	+	+	-
Bacillus circulans	+	+	+	-	+	-	-	+	+	-
Micrococcus roseus	+	+	+	-	+	-	+	-	+	-

	Starch hydrolysis rate (mm)[b]		
	Halo zone (mm)	Diameter of colony (mm)	SHR
Bacillus megaterium	16	3	5.33
Bacillus subtilis	10	2	5.0
Bacillus cereus	12	3	4.0
Bacillus thuringiesis	17	3	5.67
Bacillus lentus	14	4	3.5
Bacillus mycoides	4	2	2.0
Bacillus alvei	18	3	6.0
Bacillus polymyxa	7	5	1.4
Bacillus circulans	16	5	3.2
Micrococcus roseus	10	5	2.0

[a]: Morphological and biochemical tests used for identification of the isolated bacteria. +: Positive, -: Negative, [b]: starch hydrolysis rate.

Table 3(on next page)

Antibacterial activity of *Bacillus sp*. and purified amylase enzyme compared with different standard antibiotics.

[a]: Antimicrobial susceptibility of a group of standard antibiotics according to CLSI, 2017 against five human pathogenic strains as control. R= Resistant, S= sensitive, I= intermediate, ND= Not detected, [b:] antimicrobial activity of the isolated *Bacillus sp.,* NI= No inhibition, [c:] antimicrobial activity of purified amylase from some isolated *Bacillus sp*., ABM = amylase purified from *Bacillus megaterium*, ABS amylase purified from *Bacillus subtilis*, ABC= amylase purified from *purified Bacillus cereus*, ABT= amylase purified from *Bacillus thuringiesis*, ABL= amylase purified from *Bacillus lentus*, ABA amylase purified from *Bacillus alvei*. Values expressed as mean± SD.

Table (3): Antibacterial activity of *Bacillus sp*. and purified amylase enzyme compared with different standard antibiotics.

Tested pathogens / Antibiotic[a]/Isolates	*E. coli*	*Klebsiella pneumoniae*	*Acinetobacter baumanii*	*Pseudomonas aeuroginosa*	*Staphylococcus aureus* (MRSA)
Chloramphenicol	S	R	S	I	I
Oxacillin	ND	ND	ND	ND	R
Vancomycin	ND	ND	ND	ND	R
Ampicillin- sulbactam	I	I	R	I	ND
Penicillin G	ND	ND	ND	ND	R
Erythromycin	ND	ND	ND	ND	I
Sulfamethoxazole-trimethoprim	R	R	R	R	I
Cefotaxime	R	R	R	R	R
Gentamycin	R	R	R	R	R
Meropenem	S	R	R	R	ND
Piperacillin	R	R	R	R	ND
Piperacillin-tazobactam	R	R	R	S	ND
Inhibition zone (mm)[b]					
Bacillus megaterium	21±1.5	26±1	36±1	24±1	31±1
Bacillus subtilis	12±1	18±2	21±1	15±1.5	20±2
Bacillus cereus	12±1.2	21±1	36±1	18±1	14±1.5
Bacillus thuringiesis	14±1.5	21±1	21±1	22±1	18±0.6
Bacillus lentus	22±1.6	22±1	24±0.6	32±1.5	31±3
Bacillus mycoides	NI	NI	NI	NI	NI
Bacillus alvei	34±1.5	21±1	39±1.5	29±2.5	48±2
Bacillus polymyxa	36±2.5	17±0.6	38±1	17±4	20±0.6
Bacillus circulans	21±1.5	23±1	39±1	32±1	32±2
Micrococcus roseus	NI	NI	NI	NI	NI
Inhibition zone (mm)[c]					
ABM	7.2+0.3	7.5+0	NI	NI	NI
ABS	7.3+0.3	7+0	NI	NI	NI
ABC	7+0.3	7.3+0.3	NI	NI	NI
ABT	7.5+0	7.2+0.3	NI	NI	NI
ABL	7.3+0	7.5+0	NI	NI	NI
ABA	7.5+0.5	7.3+0.3	NI	NI	NI

[a]: Antimicrobial susceptibility of a group of standard antibiotics according to CLSI, 2017 against five human pathogenic strains as control. R= Resistant, S= sensitive, I= intermediate, ND= Not detected, [b]: antimicrobial activity of the isolated *Bacillus sp.,* NI= No inhibition, [c]: antimicrobial activity of purified amylase from some isolated *Bacillus sp*., ABM = amylase purified from *Bacillus megaterium*, ABS amylase purified from *Bacillus subtilis*, ABC= amylase purified from *purified Bacillus cereus*, ABT= amylase purified from *Bacillus thuringiesis*, ABL= amylase purified from *Bacillus lentus*, ABA amylase purified from *Bacillus alvei*. Values expressed as mean± SD.

Table 4(on next page)

Purification profile of amylase produced from different isolates of *Bacillus* sp.

[a]: different purification steps of amylase purification, [b:] selected isolated *Bacillus sp*. for amylase purification according to SHR, EA: enzyme activity, TA: total activity

Table (4): Purification profile of amylase produced from different isolates of *Bacillus* sp.

Purification step[a] / Isolates[b]	Crude		Ammonium sulfate		Dialysis		Sephadex G-200	
	EA (U/ml)	TA (U)	EA (U/ml)	TA (U)	EA (U/ml)	TA (U)	EA (U/ml)	TA (U)
Bacillus megaterium	33.15	6630	35.06	701.0	35.9	718	80.03	800
Bacillus subtilis	25.0	5000	32.0	640	35.0	700	76.0	760
Bacillus cereus	19.66	3932	23.08	461.0	25.12	502	55.9	559
Bacillus thuringiesis	39.45	7890	41.0	820	40.26	805	88.64	886
Bacillus lentus	13.9	2780	15.4	308	18.26	365	45.69	456.9
Bacillus alvei	50.2	10040	51.47	1029	29.56	591	96.02	960

[a]: different purification steps of amylase purification, [b]: selected isolated *Bacillus sp*. for amylase purification according to SHR, EA: enzyme activity, TA: total activity

Figure 1

Antagonistic efficacy of isolated *Bacillus sp*. from soil against some human pathogenic bacteria.

(a) represent moderate inhibition, (b) represent the highest inhibition, (c) represents no inhibition (d) represent starch hydrolysis rate (SHR).

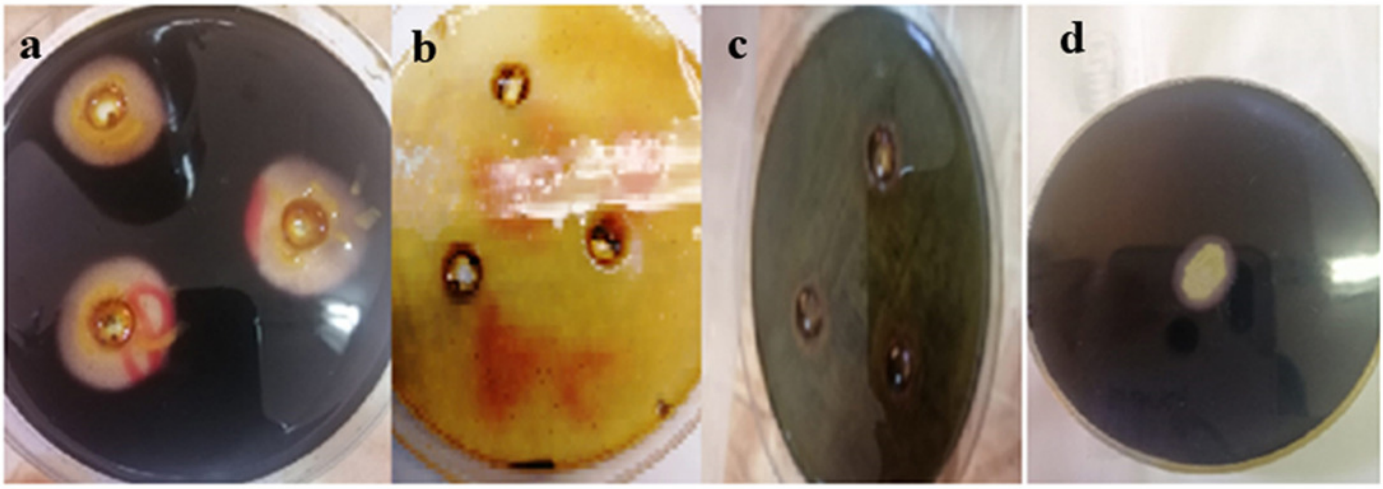

Figure 2

Optimization and purification conditions of amylase enzyme from selected *Bacillus sp*.

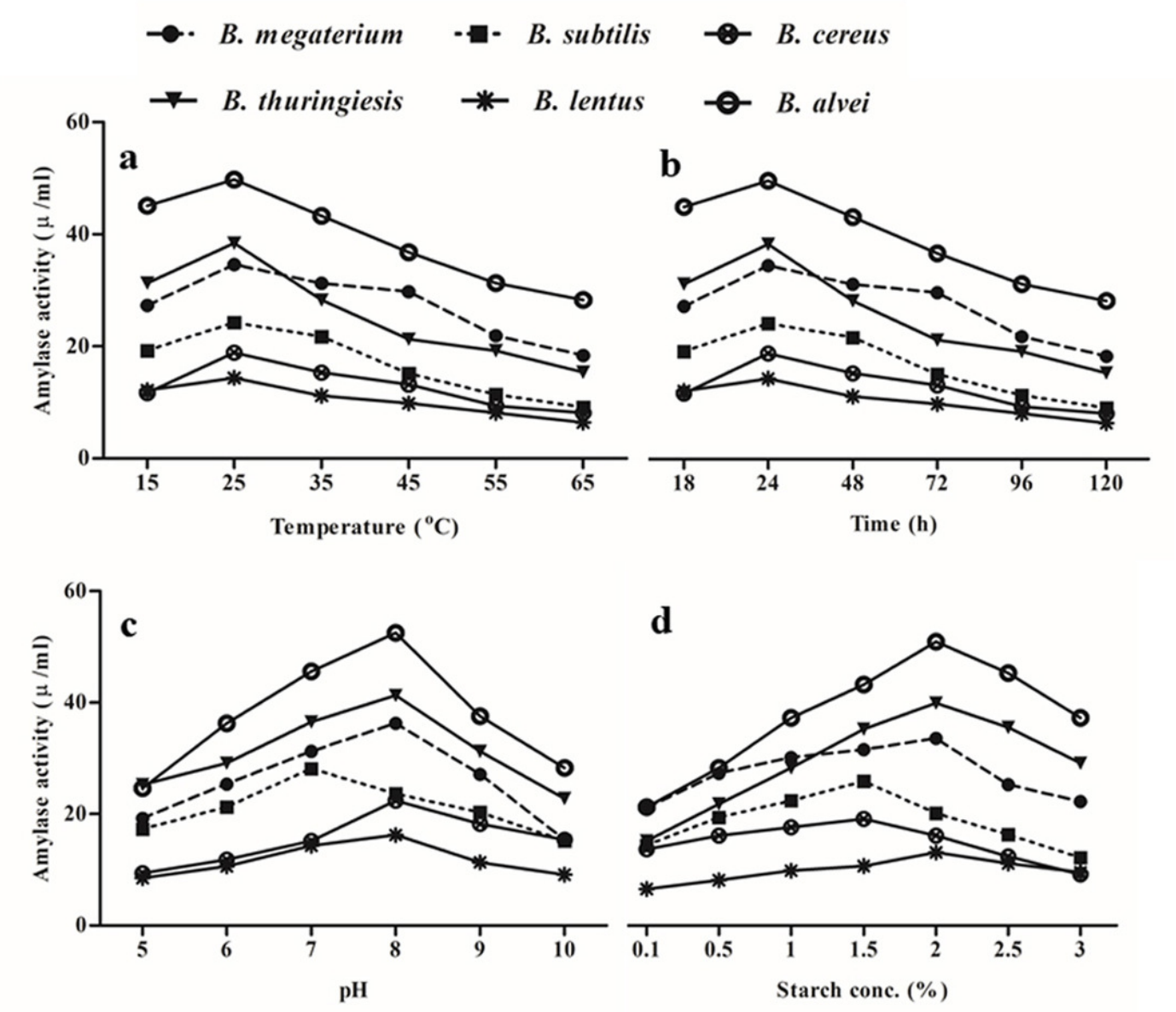

Figure 3

Antibiofilm activity of *Bacillus*-producing amylase-filtrate against some pathogenic bacteria after 48h treatment.

Shown are *Bacillus* sp. filtrate, T1: *B. megaterium*, T2: *B. subtilis*, T3: *B. cereus*, T4: *B. thuringiesis*, T5: *B. lentus*, T6: *B. alvei*, T7: *B. polymyxa*, T8: *B. circulans*. The tested pathogenic bacteria are (a) *E. coli*, (b) *Klebsiella pneumoniae*, (c) *Acinetobacter baumanii*, (d) *Pseudomonas aeruginosa,* (e) *Staphylococcus aureus* (MRSA). Shown are the medians from at least eight independent measurements. The error bars indicate the interquartile range.

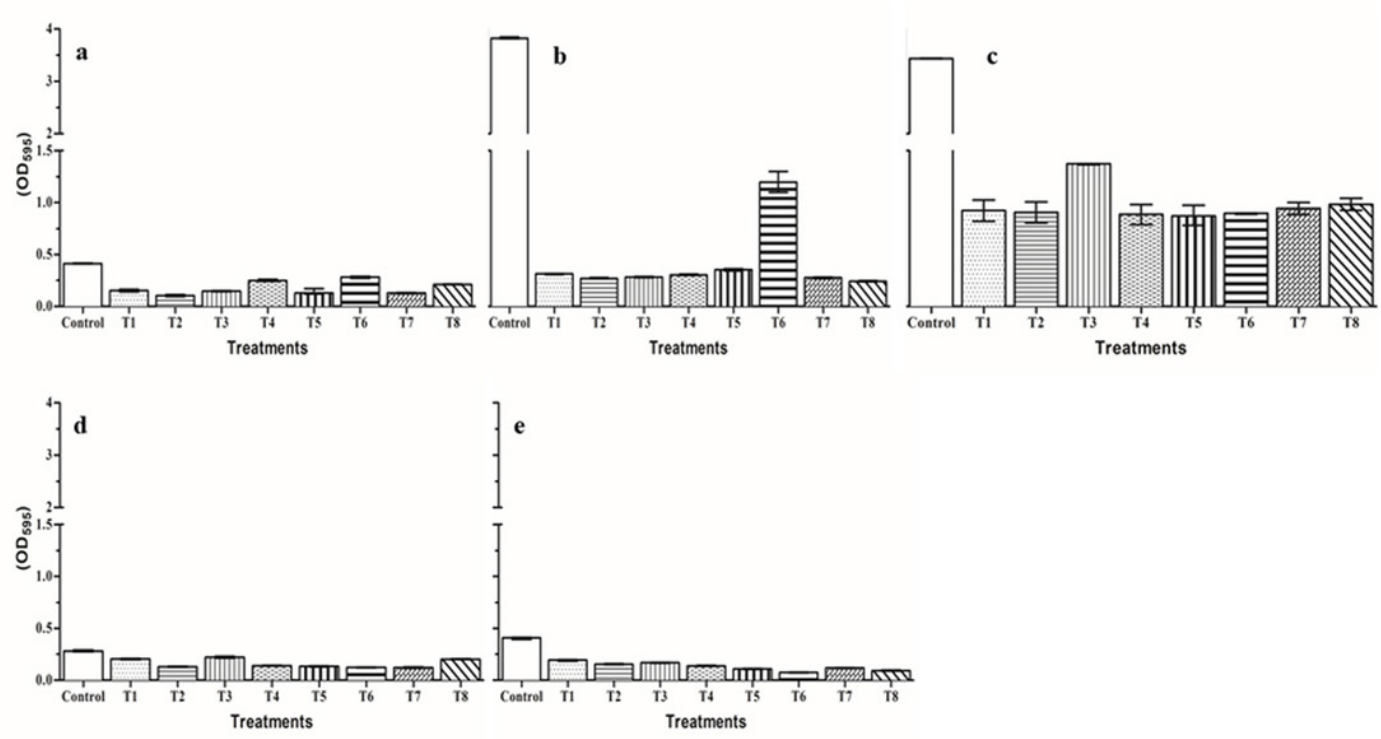

Figure 4

Antibiofilm activity of *Bacillus*- purified amylase enzyme against some pathogenic bacteria after 48h treatment.

Shown are *Bacillus* sp. filtrate, T1: *B*. *megaterium*, T2: *B. subtilis*, T3: *B. cereus*, T4: *B. thuringiesis*, T5: *B. lentus*, T6: *B*. *alvei*. The tested pathogenic bacteria are (a) *E. coli*, (b) *Klebsiella pneumoniae*, (c) *Acinetobacter baumanii*, (d) *Pseudomonas aeruginosa*, (e) *Staphylococcus aureus* (MRSA). Shown are the medians from at least eight independent measurements. The error bars indicate the interquartile range.