Antibiofilm activity of amylase from *Bacillus* spp. against foodborne pathogens

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Abstract

New strategies have been currently developed for removal and control of biofilm-associated microbial infections. Therefore, it is aimed to research the antibiofilm activity of amylase from Bacillus spp. on biofilm forming Citrobacter spp. 1.3 and 2.5 foodborne pathogens in this study. The bacteria belonging to genus Bacillus were isolated from Kilis soil sample. These strains produced amylase were named as 2B, 5B and 8B.

Compared to others, the extracellular amylase sample purified from 2B strain showed the highest specific activity (20.83±0.77 U mg^{-1}) at 90 mg mL^{-1} enzyme concentration. Molecular weight of the amylase was calculated as 45 kDa by SDS-PAGE and native PAGE. The pink colonies of foodborne pathogens indicating antibiofilm activity of amylase were followed on congo red agar during 6-24h incubation. Our results revealed that 2B amylase sample at the 90 mg mL^{-1} concentration effectively inhibited the synthesis of exopolysaccharides of biofilm forming pathogens.

Keywords: Amylase, antibiofilm, *Bacillus* spp., foodborne pathogens.

Introduction

Biofilm, self-produced by microorganisms in many biotic and abiotic surfaces such as clinical, natural and infrastructural environments, is a three-dimensional gelatinous slimy matrix[23]. This matrix known as extracellular polymeric substance (EPS) consists of polysaccharides, proteins, DNA and one or more microbial (bacterial or fungal) species[24]. The unique lifestyle having structural stability protects microorganisms against harsh environmental conditions such as host immunological system, phages and antimicrobial agents[19, 23].

Biofilm causes various clinic infections that are especially associated with the use of intravascular and urethral catheters of orthopedic devices, contact lenses, prosthetic heart valves, vocal cord prosthesis[16]. The spread of resistance against antibiotics used in medical field is also linked to microbial biofilm formation[18].

Some physical/mechanical treatments such as ionizing radiation, bioelectric effects, ultraviolet radiation and ultrasonication were performed for removal of biofilm[19]. But, the newer stratiges targeting biofilm structure for resistance suppression have been developed in recent years[23].

Enzyme use is one of the newer approaches for biofilm distruption. Antibiofilm enzymes target to continuously separate the cells breaking down the cell-to-cell linkage of cells embedded in the matrix. This accelerates the degaradation of the matrix macromolecules. The proteolytic enzymes generally degrade chemical bonds between macromolecules of EPS. And this substrate-specific activity can changes, varying from species to species of EPS compositions[5]. In this study, we aimed to investigate the potential antibiofilm activity of amylase identified from *Bacillus* spp. against foodborne pathogens produced biofilm.

Material and Methods

Isolation of amylase-producing bacteria: *Bacillus* spp. were isolated from soil sample collected at Kilis, Turkey. Following suspended by sterile water (1:9), soil sample was incubated at 65ºC for 30 min. for stimulation of bacterial spores. 100 µL of this culture was inoculated by using serial dilution technique on nutrient agar and incubated at 37°C for 24 h. Single colony was selected for amylase activity test. For screening amylase activity, cells were grown on agar medium (including starch 5.0 g, tryptone 10.0 g, yeast extract 5.0 g, NaCl 10.0 g, agar 15.0 g, per liter pH 7.0±0.2) at 37°C up to 24 h.

Then plates were overlaid with iodine reagent [0.33% (w/v) iodine and 0.67% (w/v) potassium iodide][11] and clear zones indicated the hydrolization of starch by bacteria observed. Amylase producing bacteria were identified by applying morphological (Gram staining, spore forming and cell morhpology) and standard microbiological (catalase test systems) procedures.

Extraction of amylase from *Bacillus* spp.: For preparation of bacterial inoculum, strains were grown using Luria-Bertani media during overnight at 37°C. Subsequently, 5 mL of inoculum (McFarland 0.5) was transferred into amylase broth consisting of peptone 10.0 g, starch 20.0 g, yeast extract 4.0 g, $CaCl_2$ 0.2 g and $MgSO_4$ 0.5 g, pH 7.0±0.2[8]. Cultures were incubated at 37ºC, 180 rpm for 24 h. The cells were harvested by using centrifugation at 5500 rpm for 20 min. after incubation.

Then, the obtained supernatant was lyophilized (Teknosem, Toros TRS 2/2V) and evaulated as crude enzyme sample. This crude amylase was stored at -20ºC for further analyses

(enzyme activity, total protein concentration, protein molecular weight and antibiofilm activity).

Amylase activity analysis: Freeze dried enzyme samples were suspended at 0.3-0.9 mg mL^{-1} concentrations in 50 mM phosphate buffer (pH 7.0). Enyzme activity was measured based on reaction reducing sugars liberated as a result of amylase activity with 3,5 dinitrosalicylic acid (DNS) reagent. The crude enzyme (50 µL) was added to 500 µL of substrate solution (1% starch in 50 mM sodium phosphate buffer, pH 7.0) and 450 µL of sodium phosphate buffer.

This mixture was incubated at room temperature for 5 min. Then, the reaction was stopped by adding 1 mL of 3,5 dinitrosalicylic acid (DNS) reagent (0.3 g DNS; 9 g sodium potassium tartarate tetrahidrate; 6 mL 2N NaOH; 9 mL distilled water). For monitoring of color development, the tubes were heated at 95ºC for 10 min. 5 mL distilled water was added to tubes after the orange-red color forming.

The absorbance was spectrophotometrically meausured at 546 nm against a blank. A calibaration curve was ploted using glucose standards (0-5 µmol mL^{-1}). A unit of enzyme activity was calculated as the amylase amount needed to release 1 µmol of sugar reduced per minute. Activity analysis for all enzyme concentrations was performed in triplicate. Total protein concentration of crude enzyme was detected by Lowry et al[15] method.

SDS-PAGE analysis: Molecular weight of amylase was determined according to the Laemmli[13] procedure. ABM (Applied Biological Materials) opti-protein G252 was used as reference marker. Protein bands were monitored by staining Coomassie Brilliant Blue R-250. Amylase activity on native PAGE was searched by using solution prepared without denature agents. 3% starch (w/v) was co-polymerized with separating gel. After electrophoresis, the gel was stained with iodine reagent. Clear zones around protein bands on the blue background are attributed to the amylase activity.

Biofilm removal activity of amylase: For antibiofilm researches, biofilm producing foodborne pathogens previously isolated in lab. were used. These bacteria were identified as *Citrobacter* spp. 1.3 and 2.5. Pathogens were grown in nutrient broth at 37°C for 24 h. The density of overnight cultures was adjusted to 0.5 McFarland turbidity. 250 µL of these suspension and 750 µL of purified amylase enzyme showing the highest specific activity were added to tubes including 1 mL nutrient broth. A loopfull sample from overnight culture of test tubes was inoculated to congo red agar (brain heart infusion broth (BHI) supplemented with 5% sucrose and 0.8 g L^{-1} congo red stain :CRA)[17]. After 24h incubation, the colors of colonies on plates were observed. The dark red or blackish colonies and pink colonies were evaluated as biofilm forming and biofilm distruption respectively[14]. The color change in colonies was observed during 6-24 h.

Results and Discussion

Totally, 20 *Bacillus* strains were isolated from Kilis soil sample. For exhibition of amylase production, all strains were inoculated on starch agar medium. Among them, only three isolates having amylase activity were selected for amylase extraction analysis (Figure 1). Similar findings related to amylase activity in agar plate were reported by many researchers in the literature[2,21]. These isolates showed positive results for grams staining, spore forming, catalase and amylase test, were entitled as 2B, 5B and 8B.

Figure 1: Amylase activity (zone of clearance) of *Bacillus* spp. on starch agar

The crude amylase enzymes were partially purified from supernatants of *Bacillus* cultures and lyophilized. The specific activity of concentrated amylase samples is presented in table 1. The variation was observed in specific activity of extracellular amylase purified from different *Bacillus* strains.

Generally, the enzyme activity were determined to increase depending upon the increase of concentration. The lowest and highest enzyme activity was recorded at the 30 mg mL^{-1} and 90 mg mL^{-1} concentration for all samples respectively.

Among the tested concentrations, the lowest activity was calculated in 8B sample (13.44±0.66 U mg^{-1}) compared to the other amylase preparation. The specific activity increased from 15.88±0.58 to 16.11±0.88, 16.34±0.58 and 20.83±0.77 U mg^{-1} with increasing concentrations of enzyme for 2B amylase sample. The highest amylase activity was 20.83±0.77 U mg^{-1}.

Similarity, the activity of 5B amylase sample increased from 15.59±0.65 to 20.21±0.99 U mg^{-1} depending on concentration increase. The specific activity at the wide range for amylase enzyme among the various *Bacillus* sp. was reported. Raul et al[22] declared that the enzymatic activity of amylase from Bacillus subtilis was 13.14 (μmol/mg/min).

Table 1
Specific activity of amylase at the different concentrations purified from *Bacillus* spp.

	30 mg mL^{-1}	**50 mg mL^{-1}**	**70 mg mL^{-1}**	**90 mg mL^{-1}**
	Specific activity (U mg^{-1})			
2B	15.88±0.58	16.11±0.58	16.34±0.88	20.83±0.77
5B	15.59±0.65	17.30±0.76	17.79±0.16	20.21±0.99
8B	13.44±0.66	15.22±0.40	16.85±0.28	17.14±0.75

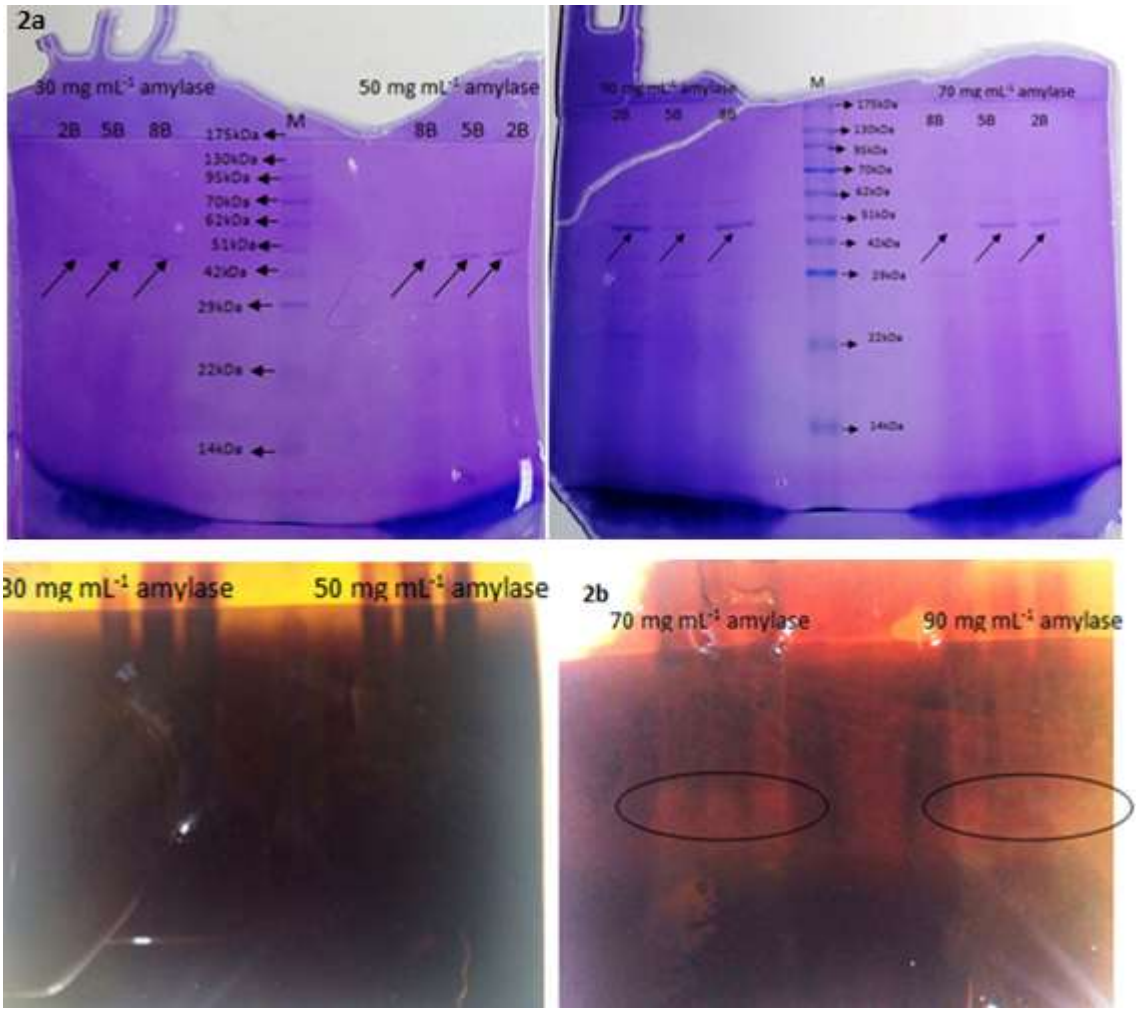

Figure 2: (a) 12%SDS gel profile for amylase samples at the different concentrations, M: Marker (b) Zymogram for amylase sample on native PAGE

In the other study, Kiran et al[12] indicated to reach the maximum specific activity (2.11 U mg^{-1}) of amylase from *Bacillus* sp. K12 after 60 h.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular weight of amylase was carried out. As seen in figure 2a, the crude enzyme samples have many protein bands with different molecular weights. The molecular weights of the bands were ranging from 31.5 to 56 kDa. To determine protein band belonging to amylase, hydrolytic activity of the enzyme on native PAGE supported starch was investigated.

In lanes of the amylase-enriched samples (70 and 90 mg mL^{-1}), the hydrolytic activity were observed as a clear band in dark background due to iodine reagent (Figure 2b). Only one protein band (approximately 45 kDa) indicating the presence of extracellular amylase was recorded. Absence of this clear band for samples at the 30 and 50 mg mL^{-1} concentrations demonstrated to the insufficient amount of amylase interacted with substrate. These findings of molecular mass exhibited similarity with previous study: 42 kDa for *Bacillus cereus*[1] and 42.8 kDa for *Bacillus subtilis* DM-03[7].

Many researchers notified different molecular mass of amylase purified from *Bacillus* sp: 56 kDa for *Bacillus subtilis* KIBGE HAS[3]; 47 and 67 kDa for *Bacillus subtilis* X-23[20]; 31 kDa for *Bacillus licheniformis*[4] and 67 kDa for *Bacillus tequilensis* RG-01[25].

Antibiofilm activity was performed by using amylase sample having the highest activity. So, the 2B enzyme sample at the 90 mg mL^{-1} concentration was tested. The antibiofilm activity followed by CRA method is demonstrated in figure 3a-b. The colony color of *Citrobacter* spp. 2.5 was pink during 24h incubation. This antibiofilm effect of 2B amylase was observed against *Citrobacter* spp. 1.3 after 6 and 12 h of incubation. But colony color of 1.3 strain was changed from pink to black after 24 h. So, the congo red analysis treated with 90 mg mL^{-1} 2B amylase against 1.3 strain indicated the reduction in antibiofilm activity after 24h. These results showed that 2B amylase sample at the 90 mg mL^{-1} concentration effectively inhibited the synthesis of exopolysaccharides of biofilm forming pathogens (Figure 3b).

Similar results relating to EPS degradation by amylase enzyme from *Bacillus* sp. were noted in previous studies. The antibiofilm activity of extracellular α-amylase from *Bacillus subtilis* S8-18 against clinic pathogens was reported by Kalpana et al[10]. Gomma[9] revealed the biofilm inhibiton of amylase from *Bacillus subtilis* and *Bacillus cereus* against *Klebsiella* sp.

In the other study, Craigen et al[6] indicated the effective biofilm reduction property of *α*-amylase purified from *Bacillus subtilis*. The main mechanism of antibiofilm activity explained that the hydrolytic enzyme weakened the physical structure of EPS by destroying the bonds of the proteins, carbohydrates and lipids and subsequently suppressed the biofilm formation process. Especially, the disruption of EPS matrix in biofilm associated infection accelerates the penetration effect of drugs applied against target bacterial cells.

Conclusion

In this study, we have showed the production and purification of amylase enzyme from soil bacteria *Bacillus* spp.. The succesful antibiofilm activity of 2B amylase enzyme was also indicated against *Citrobacter* spp. 1.3 and 2.5. Our results suggested that this amylase enzyme may be developed as biofilm inhibitor agent in the new applications for biofilm distruption.

We will continue to investigate potential effects of the combination studies with different hydrolytic enzymes for completely biofilm removal in further analysis. And this antibiofilm potential of 2B amylase will be confirmed by anti-adhesive (biofilm formation inhibition) and Scanning electron microscopy studies.

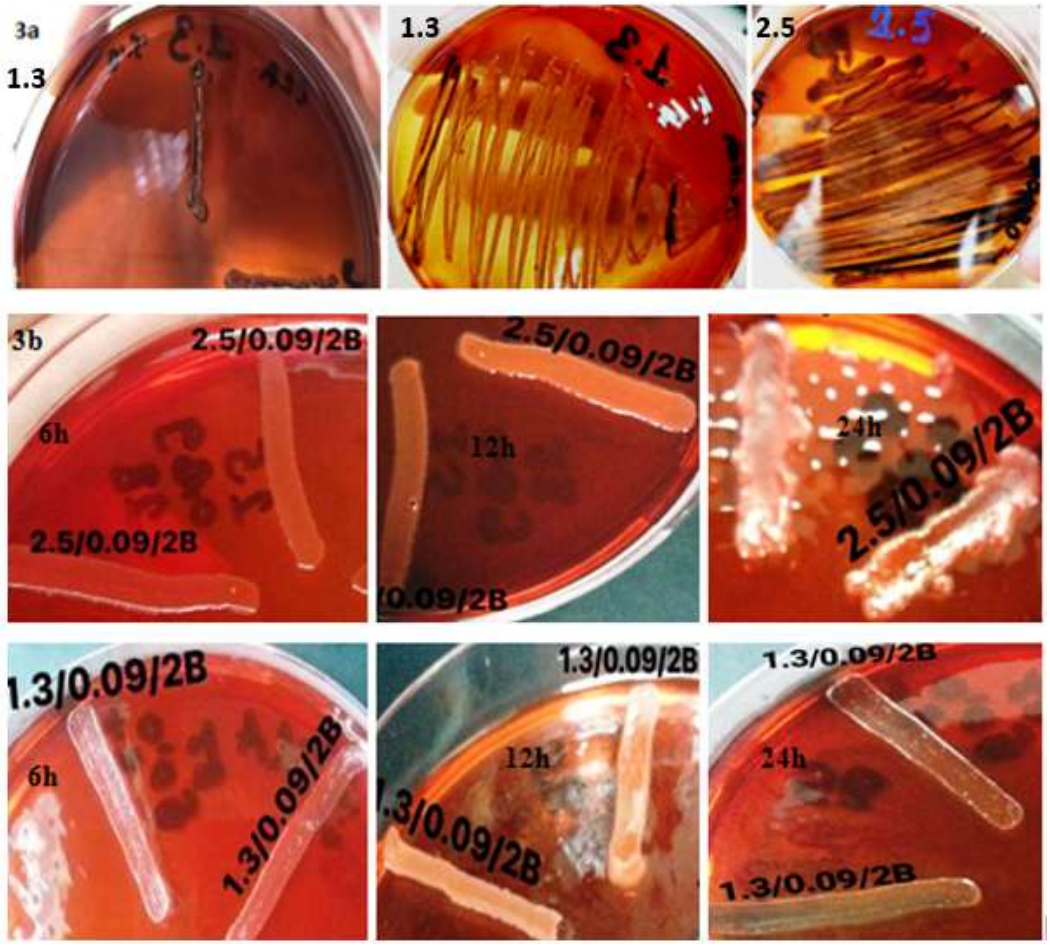

Figure 3: (a) Biofilm formation potential (black colony) of *Citrobacter* spp. 1.3 and 2.5 strains (b) Congo red assay: Biofilm inhibiton with 90 mg mL^{-1} 2B amylase during 6, 12 and 24 hours incubation at 37°C.

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