Journal of Agricultural Faculty of Uludag University, 2016,
Volume: 30, Number: Special Issue, 214-222

Isolation of Chitinase Producing *Bacillus* Strains and Increasing of the Enzyme Production by *in vitro* Mutagenesis

Burcu DURMAZ[1] and Bahri Devrim ÖZCAN[2*]

[1] Osmaniye Korkut Ata University, Graduate School of Natural and Applied Sciences, Department of Biology, 80000, Osmaniye, TURKEY
[2] Osmaniye Korkut Ata University, Faculty of Arts and Sciences, Department of Biology, 80000, Osmaniye, TURKEY
* Corresponding author: devrimozcan@osmaniye.edu.tr

ABSTRACT

In the present study, three chitinolytic *Bacillus* strains were isolated from the soil samples containing shells of crustaceans collected from the coast sediments of Erzin district of Hatay province. The isolates were entitled as *Bacillus* sp. DBK1, DBK2, and DBK3, respectively. The optimum enzyme activities were observed at 40 °C for all chitinases. On the other hand, optimum pH value for DBK1 chitinase was 7.0, whereas it was 6.0 for DBK2 and DBK3 chitinases. The specific activities of DBK1, DBK2, and DBK3 chitinases were 6.61, 13.17 and 8.65 U/mg at 40 °C, respectively. Maximum enzyme productions of isolates were observed after 48, 36, and 60 hours after inoculation of DBK1, DBK2, and DBK3 isolates, respectively. Mutant varieties DBK1-M3 and DBK1-M4 from DBK1, DBK2-M3, DBK2-M4 and DBK2-M5 from DBK2, and DBK3-M3 and DBK3-M4 from DBK3 were obtained after ethidium bromide (EtBr) treatment. DBK1-M3, DBK1-M4, DBK2-M3, DBK2-M4, DBK2-M5, DBK3-M3 and DBK3-M4 have produced 146, 193, 192, 121, 136, 178 and 116% more chitinase according to their own wild type strains, respectively. The activators for DBK1 chitinase were $MnCl_2$, $MgCl_2$, $CaCl_2$, $ZnCl_2$, and $CuSO_4$, for DBK2 chitinase were $MnCl_2$, $ZnCl_2$ and KCl, and for DBK3 chitinase were $MnCl_2$, $CaCl_2$ and $ZnCl_2$. $HgCl_2$ strongly inhibited all wild type chitinases. These properties of the enzymes indicated that they could be used as an enzyme supplement for aerobic stability of silage.

Keywords: *Bacillus* sp., Chitinase, Isolation, Characterization, Mutation, Silage aerobic stability

INTRODUCTION

Chitins are a group of linear polymers of β-1,4-linked N-acetyl-D-glucosamine (GlcNAc) units (Anuradha and Revathi 2013). Its chemical structure is similar to the cellulose with a minor difference, that one of the hydroxyl groups of glucoside residues is replaced by an amino group (Kuddus and Ahmad 2013). These polymers are widely distributed in nature as the structural components of insects, crustaceans, cell walls of most fungi and algae (Kuddus and Ahmad 2013, Wang and Chang 1997). Chitin is the second most abundant natural polymers and possibly the most abundant in the marine environment (Svitil *et al*. 1997).

Chitinases (EC 3.2.1.14) are enzymes that catalyse the conversion of chitin to its monomeric or oligomeric compounds (Kuzu *et al*. 2012). A wide range of organisms such as bacteria, fungi, plants, insects, crustaceans and vertebrates produce chitinases (Tasharrofi *et al*. 2011). Most bacteria and fungi species produce chitinase to convert chitin into carbon and nitrogen for energy source (Natsir *et al*. 2010). Nevertheless, it was thought that plants produce chitinases to protect themselves from chitin-containing parasites with some direct evidence supporting the lacking of this hypothesis (Verburg and Huynh 1991). In commercial applications, chitinases can be used in controlling of pathogenic fungi in plants and insects, production of chitin oligosaccharides, production of single cell protein, preparation of mycolytic enzymes, and the formation of fungal protoplasts in biotechnological applications (Fleuri *et al*. 2009).

The aim of this study was to isolate new *Bacillus* species from soil samples containing shells of crustaceans, and partial characterization of the strains and enzymes. Additionally, increasing of enzyme productions by isolates using *in vitro* mutagenesis method was also studied.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The soil samples were collected from coast sediments of South Turkey. One gram of soil sample was suspended in 10 mL of deionized and sterilized water. After the pasteurization (15 min, 80 ºC), 100 µL of suspension was spread on colloidal chitin agar (g L -1: bacto tryptone, 10; yeast extract, 5; NaCl, 10; colloidal chitin, 10; agar, 15; pH 7.5) plates and incubated for 72 hours at room temperature. The chitinolytic bacteria were selected according to their clear activity zones.

Preparation of colloidal chitin

Colloidal chitin was prepared from the chitin (Sigma, C9752) using the method of Kuddus and Ahmad (2013) with few modifications. Forty grams of commercial chitin were added slowly into 600 mL of concentrated HCl and stirred vigorously at 30 ºC. The mixture was added into 2 litres of purified water and stirred vigorously at 4-10 ºC. The precipitate (colloidal chitin) was collected by filtration on Whatman no 5. The suspension was washed by 5 litres of distilled water and collected again by filtration on Whatman no 5. This step was repeated three times until the pH of suspension was reached to approximately 3.5. The prepared colloidal chitin was used as substrate for further studies.

Purification of chitinases from isolates

Chitinase enzymes in the culture supernatants of isolates were precipitated with ethyl-alcohol (Et-OH) method according to Kuzu *et al* (2012). Ice-cold Et-OH was added 70% of the original volume of supernatants and the mixtures were incubated in -20 ºC condition for 24 hours. The precipitates were recovered by centrifugation (10,000 rpm (4 ºC), 30 min), dissolved in 0.1 M Na-phosphate buffer (pH 7.0), and stored at 4 ºC until usage.

Enzyme assay

The chitinase activities were measured using chitin-azure as substrate. Reaction mixtures including 1 mL of enzyme solution, 1 mL of sodium phosphate buffer (50 mM, pH 6.5) and 2.5 µL of chitin-azure solution (2 mg mL -1 chitin-azure in sodium phosphate buffer (50 mM, pH 6.5), stored in dark bottle at 4 ºC) were incubated at 37 ºC for 30 min. Enzyme activities were measured at 595 nm absorbance using spectrophotometer (Kuzu *et al*. 2012). Each experiment was repeated for three times and data were averaged. One unit of chitinase activity was the amount of enzyme which yields 1 µmol of reducing N-acetyl-D-glucosamine (GlcNAc) equivalent per minute under the conditions of this study.

pH and temperature optima

The optimum pH and temperature values of the enzymes were determined by incubation in a buffer at different pH values (5.0-13.0) and temperatures (30-100 ºC) under standard assay conditions using chitin-azure as the substrate. The buffers used were 50 mM citrate buffer (pH: 4.0–6.0), 50 mM sodium phosphate buffer (pH: 6.0–8.0), 50 mM Glycine-NaOH buffer (pH: 8.0–10.0), and 50 mM borax-NaOH buffer (pH: 10.0–13.0). The optimum temperature for enzyme activity was measured under standard assay in the range of 30–100 ºC with intervals of 10 ºC (Kuzu *et al*. 2012).

The effect of temperature and pH on stability of the enzymes

The effect of the temperature on the stability of enzymes was determined by exposure of the 1 mL of enzyme solutions in 1 mL of 50 mM Na-phosphate buffer (pH 7.0 for DBK1 enzyme, pH 6.0 for DBK2 and DBK3 enzymes (optimum pH values)), to different temperatures (30-100 ºC) for 30 min. The residual activities were than determined under standard conditions (Kuzu *et al*. 2012).

For pH stability assay, the enzyme solutions were pre-incubated for 30 min at 40 ºC in the buffers of different pH values (7.0-13.0). The residual activities were than determined under standard conditions (Kuzu *et al*. 2012).

Time dependent enzyme productions by the strains

A single colony of each strain was inoculated in 10 mL LB broth and incubated at 37 ºC for 24 h. After incubation, 0.5 mL cultures were inoculated in 50 mL LB broths and incubated at 37 ºC for 72 h. Culture samples were collected and centrifuged at 4.900 rpm for 10 minutes in times 12 h, 24 h, 36 h, 48 h, 60 h and 72 h to measure the enzymatic activities under standard conditions.

The effect of chemicals on enzyme activities

The chemicals were used in 1 mM concentrations. The effect of these chemicals on enzyme activities were determined by incubating 1 mL enzyme with 1 mM EDTA, HgCl2, MnCl2, MgCl2, CaCl2, ZnCl2, KCl,

CuSO4, CoCl2 and AgNO3 for 30 min at room temperature. The residual activities were than determined under standard conditions (Kuzu *et al*. 2012).

Chemical mutagenesis of the isolates using EtBr

Mutation procedures were performed as previously described by Özcan (1996). Overnight cultured isolates in LB broth were serial diluted to a final dilution of 10^{-6}. 0.1 mL of 10^{-5} and 10^{-6} dilutions were spread on LB agar plates containing colloidal chitin by sterile glassy stick. 30 µL of EtBr (10 mg/mL) solutions were dropped on the pre-marked points on the plate with a micro-pipette. The surfaces of the plates were air-dried for 30 min and then incubated at 37 ºC for 24 h. The colonies around the toxic zones were picked onto new LB-agar plates containing colloidal chitin with sterile toothpicks. The plates were incubated at room temperature for 72 h. The plates were screened and selected for over-chitinase activities according to zone diameters. The enzymatic activities of the mutant isolates were determined under standard conditions and finally compared with each other and wild type enzymes.

RESULTS

Chitinase producing bacteria (*Bacillus* sp. DBK1, DBK2 and DBK3) were isolated from the soil samples containing shells of crustaceans and chitinases were produced in LB-agar containing 10% of colloidal chitin (Figure 1). Similarly, different species of *Bacillus* have been reported to produce chitinase (Han *et al*. 2014, Takayanagi *et al*. 1991, Waldeck 2006, Melentiev *et al*. 2014, Sarvani and Iyer 2012; Kuzu *et al*. 2012, Loni and Bajekal 2011).



Figure 1. Chitinase activities on LB-agar plates containing colloidal chitin.

pH and temperature optimum

The optimum pH value of DBK1 chitinase was 7.0, whereas 6.0 for DBK2 and DBK3 chitinases (Figure 2). The average enzyme activities between 7.0-10.0 pH values were calculated as 77.75, 45.75 and 83.50% for DBK1, DBK2 and DBK3 chitinases, respectively. However, the average activities between 4.0-7.0 pH values were 60.5, 87.25 and 73.5%, in the same order. These results indicate that, our chitinases also exhibited their activities in a wide range of pH with a neutral character.

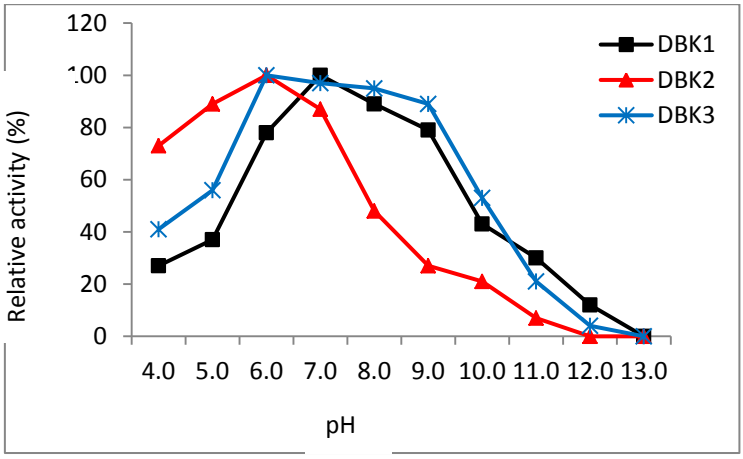


Figure 2. The effect of pH on chitinase activities.

The optimum activities were observed at 40 ºC for all chitinases (Figure 3). The average relative enzyme activities between 30-90 ºC were calculated as 48.9, 66.9 and 61.9% for DBK1, DBK2 and DBK3 chitinases, respectively. Nevertheless, the average relative enzyme activities between 50-90 ºC were 41.2, 59.8 and 49.2%, in the same order. These data indicate that although all three enzymes exhibit the mesophilic character, DBK3 chitinase shows slightly more thermostability compared to the DBK1 and DBK2 chitinases.

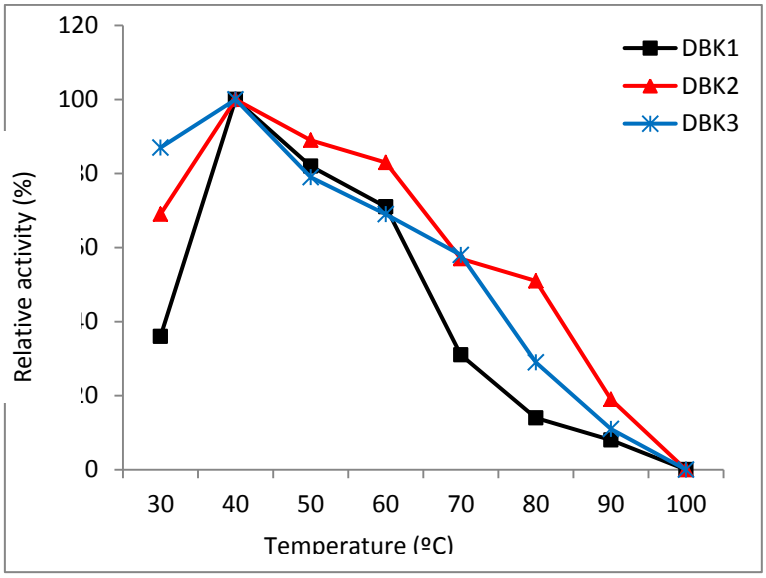


Figure 3. The effect of temperature on chitinase activities.

The chitinases produced by *Bacillus* might show different pH optimum characters. It was reported that most of chitinases are alkaline characters (Ohishi *et al*. 1996, Veda and Arai 1992), whereas others suggest that most of the chitinases are acidic (Watanabe *et al*. 1992, Yabuki *et al*. 1986, Kuzu 2008). While *B. subtilis* TV-125 (Senol *et al*. 2014) and *Bacillus* sp. (Natsir *et al*. 2002) show optimum activation at 4.0 and 5.0 pH respectively, *Bacillus* sp. R2 (Cheba *et al*. 2016), *B. circulans* No.4.1 (Wiwat *et al*. 1999), and *B. thurungiensis* subsp. HBK51 (Kuzu 2008) strain show optimum activity at 7.5, 8.0 and 9.0 pH, respectively. With a wide range of pH values, the chitinases in our study mostly showed neutral characteristics.

The chitinases from *B. circulans* No.4.1 (Wiwat *et al*. 1999), *Bacillus* sp. (Natsir *et al*. 2002), *Bacillus* sp. R2 (Cheba *et al*. 2016) *B. subtilis* BC121 (Basha and Ulaganathan 2014) showed optimum activities at 40, 37, 40 and 35 ºC, respectively. The chitinases in our study also showed similar results as aforementioned studies.

Besides, chitinases that have different optimum temperature values were also reported. Such as, the chitinase from *B. thurungiensis* subsp. HBK51 strain showed optimum activity at 110 ºC (Kuzu 2008).

Temperature and pH stability

We investigated the stability of the enzymes in response to different pH and temperature values. After pre-incubation at 50 ºC, all three enzymes retained their relative residual activities at 93, 96, 88% respectively. However, they lost most of their activities after pre-incubation at 90 and 100 ºC (Figure 4).

Khan and Khan (2014) investigated the thermal stability of the chitinases of *B. circulans* L2 and *B. licheniformis* 2J-1 strains by including the incubation period and, reported that both enzymes remained stable for 120 hours at 35-45 ºC range. Chitinases in our study also exhibited similar results by showing 97.7, 98.7 and, 96% residual activities between 30-50 ºC. Besides, it was reported that *B. thurungiensis* subsp. HBK51 chitinase remained pretty much stable at 30-120 ºC range with an average of 92.4% residual activity, retained its all activity at 90 ºC and showed 96% residual activity at 100-110 ºC range (Kuzu 2008). It should be considered as normal for *B. thurungiensis* subsp. HBK51 chitinases to show high stability at high temperatures because of the fact that its high optimum temperature value being 110 ºC.

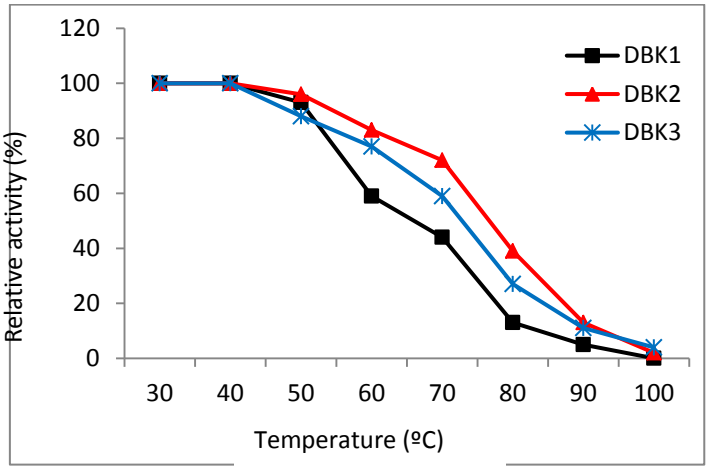


Figure 4. The temperature stability of the enzymes.

After pre-incubation between 7.0-10.0 pH values, all chitinases remained stable and retained almost all of their activities. After pre-incubation at pH 12.0, DBK1 chitinase lost 84% of its activity, whereas DBK2 and DBK3 chitinases lost almost half of their activities. In case of pre-incubation at pH 13.0, it was observed that all enzymes lost most of their activities (Figure 5). Kuzu *et al* (2012) reported that, following 3 hours of incubation period, *B. thurungiensis* HBK-51 chitinase showed a high level of activity between pH 9.0 and 12.0 (98% remaining activity) and between pH 5.0-12.0 (87% remaining activity). Likewise, it was reported that, *Bacillus* sp. BG-11 chitinase was very stable between pH 5.0-10.0 (Bhushan 2000). These results show a great similarity with our results.

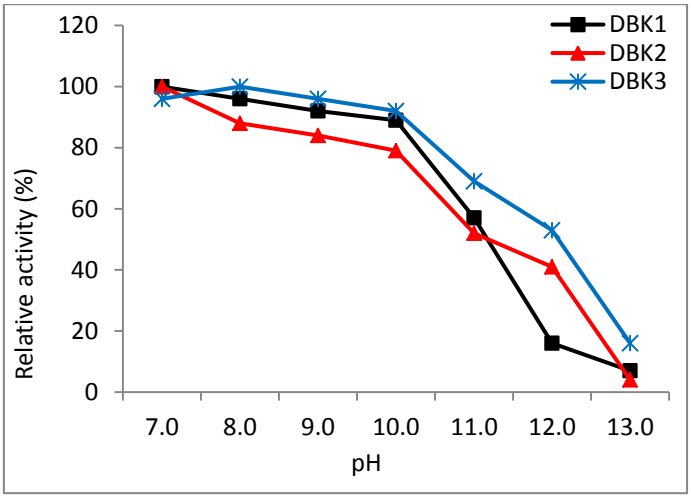


Figure 5. The pH stability of the enzymes

DBK1, DBK2 and DBK3 strains reached to maximum enzyme production levels at 48, 36 and 60 hours later from inoculation, respectively (Figure 6).

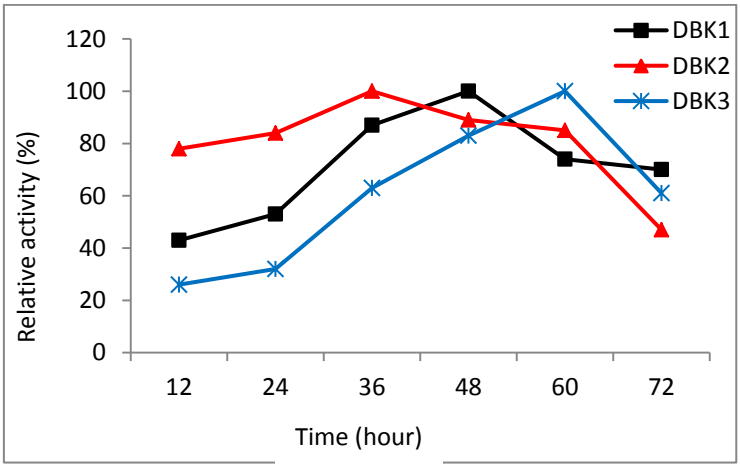


Figure 6. Time dependent chitinase productions of the isolates

The effect of chemicals on enzyme activities

The effect of various chemicals (at 1 mM concentrations) on enzyme activities were investigated (Table 1). In the presence of EDTA, $MgCl_2$, $CaCl_2$ and KCl, enzyme activities were slightly affected by between 2-12% negatively, and between 6-17% positively. On the other hand, in the presence of $HgCl_2$ and $AgNO_3$, enzyme activities were lost by 74 and 58% for DBK1, 100 and 74% for DBK2 and 93 and 67% for DBK3, respectively. In the case of presence of $MnCl_2$, enzyme activities increased with the rate of 158, 145 and 166% for DBK1, DBK2 and DBK3, respectively.

Three type interactions have been described between metal ions, enzyme and substrate. The first of these is an interaction between the substrate and the metal ion to form a complex that acts as the true substrate. The second is that the metal first binds to the protein and then serves as a site of interaction with substrate. The third scheme would have the metal acting at a site on the enzyme remote from the active site. In such instances, the metal could either serve to maintain protein structure and only influence catalytic activity indirectly or else it could regulate activity by stabilizing more or less active conformations of the protein (Riordan 1977). These complex interactions of metal ions with enzyme and substrate results in increase or decrease of the impact of the enzyme on the substrate. It was reported that effect of metal ions on chitinase activity was quite different (Yuli *et al*. 2004). Cheba *et al* (2016) reported that while K^+, Mn^{2+}, Na^+, Mg^{2+} and Ca^{2+} stimulated *Bacillus* sp. R2

chitinase activity, Cu^{2+}, Fe^{2+}, Zn^{2+}, Ag^{+} and Hg^{2+} inhibited it. Bhattacharya *et al* (2016) suggested that *B. pumilus* JUBCH08 chitinase was activated by Mg^{2+}, Co^{2+}, Ca^{2+} and Mn^{2+}, but it was strongly inhibited by Fe^{3+}, Ag^{+} and Hg^{2+}. In another study, *B. licheniformis* M3 chitinases were activated by Ca^{2+} and Na^{+}, moderately inhibited by Cu^{2+}, Co^{2+}, Zn^{2+} and, strongly inhibited by Cd^{2+} and Hg^{2+} (Jankiewicz *et al*. 2016). As our and other data indicate, metal ions have various effects on chitinase enzymes.

Table 1. Influence of chemicals on activity of the chitinases.

Chemical	Activity (%)		
	DBK1	DBK2	DBK3
Control	100	100	100
EDTA	93	93	96
$HgCl_2$	26	0	7
$MnCl_2$	158	145	166
$MgCl_2$	106	88	93
$CaCl_2$	117	94	106
$ZnCl_2$	120	119	145
KCl	98	107	97
$CuSO_4$	105	54	88
$CoCl_2$	86	67	73
$AgNO_3$	42	26	33

Mutagenesis studies

All three isolates were exposed to mutation by EtBr and quantitatively 8 mutant variants showed chitinase activity more than the wild strains (Table 2). The mutant variants DBK1-M4, DBK2-M3 and DBK3-M3 were the promising potent chitinase-producing mutants. Mutagenesis has been one of the frequent methods that are used to increase enzyme production from bacteria. With mutagenesis, overexpression of various enzymes such as chitinases (Kole and Altosaar 1985, Vaidya *et al*. 2003), amylase (Sidhu *et al*. 1997), cellulase (Pradeep *et al*. 2012), glucoamylase (Raju *et al*. 2012), xylanase (Abdel-Aziz *et al*. 2011), β-amylase (Yamashiro *et al*. 2010), α-amylase (Haq *et al*. 2010) were achieved. Similarly, we obtained mutant variants that overexpress chitinases using EtBr.

Table 2. Specific activity levels of wild type isolates and their mutant variants.

Isolate	**Mutant variants**	**Specific activity (U mg -1 protein)**	**Specific activity (%)**
Bacillus sp. DBK1	DBK1 (wild type)	6,61	100
	DBK1-M1	0,67	10.1
	DBK1-M2	4,14	62.6
	DBK1-M3	6,98	105.6
	DBK1-M4	10,23	154.77
	DBK1-M5	6,88	104.1
Bacillus sp. DBK2	DBK2 (wild type)	13,17	100
	DBK2-M1	0,21	1.6
	DBK2-M2	8,44	64.1
	DBK2-M3	19,76	150.0
	DBK2-M4	15,47	117.5
	DBK2-M5	14,5	110.1
Bacillus sp. DBK3	DBK3 (wild type)	8,65	100
	DBK3-M1	2,13	24.6
	DBK3-M2	5,07	58.6
	DBK3-M3	11,35	131.2
	DBK3-M4	9,03	104.4
	DBK3-M5	6,65	76.9

CONCLUSIONS

In this study we isolated chitinase producing wild type strains *Bacillus* sp. DBK1, DBK2 and DBK3. All three chitinases were partially characterized. After mutation by EtBr, the obtained mutant variants DBK1-M4, DBK2-M3 and DBK3-M3 were found to be more potent for chitinase production.

ACKNOWLEDGMENT

The authors are grateful to Scientific Research Projects Coordination Unit of Osmaniye Korkut Ata University for funding program.

REFERENCES

Abdel-Aziz MS, Talkhan FN, Fadel M, AbouZied AA, Abdel-Razik AS (2011). Improvement of xylanase production from *Streptomyces pseudogriseolus* via UV mutagenesis. Australian Journal of Basic and Applied Sciences, 5(5): 1045-1050.

Anuradha V, Revathi K (2013). Purification and characterization of chitinase from two *Bacillus* sp isolated from crustacean shells. Journal of Microbiology and Biotechnology Research, 3(3): 160-167.

Basha S, Ulaganathan K (2014). Identification of a broad-spectrum antifungal chitinase from *Bacillus subtilis* strain BC121. Research and Reviews: Journal of Microbiology and Biotechnology, 3(3):1-9.

Bhattacharya S, Das A, Samadder S, Rajan SA (2016). Biosynthesis and characterization of a thermostable, alkali-tolerant chitinase from *Bacillus pumilus* JUBCH08 displaying antagonism against phytopathogenic *Fusarium oxysporum*. 3 Biotech, 6(1): 87.

Bhushan B (2000). Production and characterization of a thermostable chitinase from a new alkalophilic *Bacillus* sp. BG-11. Journal of Applied Microbiology, 88(5): 453-459.

Cheba BA, Zaghloul TI, EL-Mahdy AR, EL-Massry MH (2016). Effect of pH and temperature on *Bacillus* sp. R2 chitinase activity and stability. Procedia Technology, 22: 471-477.

Fleuri LF, Kawaguti HY, Sato HH (2009). Production, purification and application of extracellular chitinase from *Cellulosimicrobium cellulans* 191. Brazilian Journal of Microbiology, 40: 623-630.

Han KI, Patnaik BB, Kim YH, Kwon HJ, Han YS, Han MD (2014). Isolation and characterization of chitinase-producing *Bacillus* and *Paenibacillus* strains from salted and fermented shrimp, *Acetes japonicus*. Journal of Food Science, 79(4): M665-M674.

Haq I, Ali S, Javed MM, Hameed U, Saleem A, Adnan F, Qadeer MA (2010). Production of alpha amylase from a randomly induced mutant strain of *Bacillus amyloliquefaciens* and its application as a desizer in textile industry. Pakistan Journal of Botany, 42(1): 473-480.

Jankiewicz U, Kozlowska M, Swiontek-Brzezinska M, Nowosielski J, Stepniewska-Jarosz S (2016). The chitinolytic bacterium *Bacillus licheniformis* M3 and its potential application in biological plant protection. Electronic Journal of Polish Agricultural Universities, 19(2): 1-5.

Khan RS, Khan ZH (2014). Studies on chitinase isolation and thermostability between *Bacillus circulans* strain L2 and *Bacillus licheniformis* 2J-1. Science Research Reporter, 4(1): 01-07.

Kole MM, Altosaar I (1985). Increased chitinase production by a non-pigmented mutant of *Serratia marcescens*. FEMS Microbiology Letters, 26(3): 265-269.

Kuddus SM, Ahmad RIZ (2013). Isolation of novel chitinolytic bacteria and production optimization of extra cellular chitinase. Journal of Genetic Engineering and Biotechnology, 11(1): 39-46.

Kuzu SB (2008). Isolation of *Bacillus* producing chitinase, partial purification and characterization of enzyme. M.Sc. Thesis, University of Çukurova, Adana, Turkey, 82p.

Kuzu SB, Korkmaz Güvenmez H, Denizci AA (2012). Production of a thermostable and alkaline chitinase by *Bacillus thurungiensis* subsp. *kurstaki* strain HBK-51. Biotechnology Research International, Article ID: 135498, 6 pages.

Loni PP, Bajekal SS (2011). Alkaline chitinase from *Bacillus firmus* SBPL-05 isolated from alkaline-saline environment of Lonar Lake. Indian Journal of Fundamental and Applied Life Sciences, 1(3): 161-165.

Melentiev AI, Galimzianova NF, Gilvanova EA, Shchelchkova EA, Kuzmina LY, Boyko TF, Usanov NG, Aktuganov GE (2014). Characterization of novel alkaliphilic isolate of *Bacillus mannanilyticus*, strain IB-OR17, displaying chitinolytic and antifungal activities. Advances in Microbiology, 4: 455-464.

Natsir H, Chandra D, Rukayadi Y, Suhartono MT, Hwang JK, Pyun YR (2002). Biochemical characteristics of chitinase enzyme from *Bacillus* sp. of Kamojang Crater, Indonesia. Journal of Biochemistry, Molecular Biology, and Biophysics, 6(4): 279-282.

Natsir H, Patong AR, Suhartono MT, Ahmad A (2010). Production and characterization of chitinase enzymes from Sulili hot spring in South Sulawesi, *Bacillus* sp. HSA, 3-1A. Indonesian Journal of Chemistry, 10(2): 263-267.

Ohishi K, Yamagishi M, Ohta T, Suzuki M, Izumida H, Sano H, Nishijima M, Miwa T (1996). Purification and properties of two chitinases from *Vibrio alginolyticus* H-8. Journal of Fermentation and Bioengineering, 82: 598- 600.

Özcan N (1996). Ethidium bromid ugulaması ile α-amilaz negatif *Bacillus subtilis* mutant suşlarının oluşturulması ve karekterizasyonu. Ç.Ü.Z.F. Dergisi, 11: 87-96.

Pradeep MR, Janardhan A, Kumar P, Narasiçha G (2012). Induction of chemical mutations in *Aspergillus niger* to enhance cellulase production. International Journal of Enviromental Biology, 2(3): 129-132.

Raju EVG, Divakar G, Swetha C, Geetha J, Satish P (2012). Strain improvement of *Aspergillus niger* for glucoamylase by physical and chemicalmutagens. International Research Journal of Pharmaceutical and Applied Sciences, 2(2): 79-91.

Riordan JF (1977). The role of metals in enzyme activity. Annals of Clinical and Laboratory Science, 7(2): 119-129.

Sarvani VS, Iyer PR (2012). Isolation, characterization and purification of chitinase from *Penicillium*, *Pseudomonas* and *Bacillus amyloliquefaciens*. Asian Journal of Microbiology, Biotechnology and Environmental Sciences Paper, 14(1): 33-38.

Senol M, Nadaroglu H, Dikbas N, Kotan R (2014). Purification of chitinase enzymes from *Bacillus subtilis* bacteria TV-125, investigation of kinetic properties and antifungal activity against *Fusarium culmorum*. Annals of Clinical Microbiology and Antimicrobials, 13: 35.

Sidhu GS, Sharma P, Chakrabarti T, Gupta JK (1997). Strain improvement for the production of a thermostable α-amylase, Enzyme and Microbial Technology, 21(7): 525-530.

Svitil AL, Chadhain SMN, Moore JA, Kirchman DL (1997). Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. Applied and Environmental Microbiology, 63(2): 408-413.

Takayanagi T, Ajisaka K, Takiguchi Y, Shimahara K (1991). Isolation and characterization of thermostable chitinases from *Bacillus licheniformis* X-7u. Biochimica et Biophysica Acta (BBA) – Protein Structure and Molecular Enzymology, 1078(3): 404-410.

Tasharrofi N, Adrangi S, Fazeli M, Rastegar H, Khoshayand MR, Faramarzi MA (2011). Optimization of chitinase production by *Bacillus pumilus* using plackett-burman design and response surface methodology. Iranian Journal of Pharmaceutical Research, 10(4): 759-768.

Vaidya RJ, Macmil SLA, Vyas PR, Chhatpar HS (2003). The novel method for isolating chitinolytic bacteria and its application in screening for hyperchitinase producing mutant of *Alcaligenes xylosoxydans*. Letters in Applied Microbiology, 36: 129-134.

Veda M, Arai M (1992). Purification and some properties of chitinase from *Aeromonas* sp. No.10S-24. Bioscience, Biotechnology, and Biochemistry, 56(3): 460-464.

Verburg JG, Huynh QK (1991). Purification and characterization of an antifungal chitinase from *Arabidopsis thaliana*. Plant Physiology, 95: 450-455.

Waldeck J, Daum G, Bisping B, Meinhardt F (2006). Isolation and molecular characterization of chitinase-deficient *Bacillus licheniformis* strain capable of deproteinization of shrimp shell waste to obtain highly viscous chitin. Applied and Environmental Microbiology, 72(12): 7879-7885.

Wang S-L, Chang W-T (1997). Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. Applied and Environmental Microbiology, 63(2): 380-386.

Watanabe T, Oyanagi W, Suzuki K, Ohnishi K, Tanaka H (1992). Structure of gene encoding chitinase –D, *Bacillus circulans* wild type 12, and possible homology of the enzyme to other procaryotic chitinase and class-3 plant chitinases. Journal of Bacteriology, 174: 408-414.

Wiwat C, Siwayaprahm P, Bhumiratana A (1999). Purification and characterization of chitinase from *Bacillus circulans* No.4.1. Current Microbiology, 39(3): 134-140.

Yabuki M, Mizushina K, Amatatsu T, Ando A, Fujii T, Shimada M, Yamashita M (1986). Purification and characterization of chitinase and chitobiose produced by *Aeromonas hydrophila* subsp. *anaerogenes*. The Journal of General and Applied Microbiology, 32: 25-38.

Yamashiro K, Yokobori S, Koikeda S, Yamagishi A (2010). Improvement of *Bacillus circulans* beta-amylase activity attained using the ancestral mutation method. Protein Engineering, Design and Selection, 23(7): 519-528.

Yuli PE, Suhartono MT, Rukayadi Y, Hwang JK, Pyun YR (2004). Characteristics of thermostable chitinase enzymes from the Indonesian *Bacillus* sp.13.26. Enzyme and Microbial Technology, 35(2-3): 147–153.