Journal of Novel Applied Sciences
Available online at www.jnasci.org
©2015 JNAS Journal-2015-4-7/824-829
ISSN 2322-5149 ©2015 JNAS

The effect of New *Bacillus* Isolate Va1-29 with Producing Carboxymethyl Cellulase (CMCase) Enzymes in Animal Nutrition

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ABSTRACT: Alkaliphilic and thermophilic *bacillus sp*. VA1-29 was isolated from Van Lake of Turkey produced thermostable CMCase at 55 °C at pH 9. The molecular weight of the mentioned enzyme was estimated to be 33 kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. working pH range of this enzyme was of 7.5-8 with an optimum pH of 7.5. The temperature optimum of the enzyme was found to be 50 °C. CMCase production by cultivated thermophilic *bacillus sp*. strain reached a maximum content at 12h which analyzed according to the Lowry method with levels of 0.6 mmol protein/min. Considering to the properties of these enzymes, the produced CMCase by *Bacillus sp*. VA1-29 can be commercialized as feed additive in food animal industrytrition.

***Keywords*:** Isolation, *Bacillus sp.*, Alkaliphilic and thermophilic , Molecular weight.

INTRODUCTION

Applying enzymes in feeds to enhance feed utilization is an idea that has been well explored in terrestrial animal feeding and to some extent in aquatic animal feeding. The primary aim of enzyme application in feeds is to improve of food digestion. It is proposed that by providing an extra dose of enzymes, the digestive processes will work better and lead to increase of feed efficiency. First related embodiment with adding of enzymes in animal feed was conducted in Finland in 1980 (21).

Importance and benefits of the use of enzymes in animal feed are improve feed efficiency, increase the quantity and quality of animal products and maintain healthy animals.

CMCase is one of important enzymes in animal nutrition. The use of enzymes in poultry nutrition which produced from barley with mixing β-glucanase is especially important in the poultry sector because it is not possible to digest of β-glucan barley without enzymes. CMCase enzymes are relatively less used in the animal nutrition. It suggest that amount of 10% use for mono-gastric species (21).

Three major types of enzymatic activities are found: A) endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (CMCase) (EC 3.2.1.4), B) exoglucanases, including 1,4-β-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (cellobiohydrolases)(CBH)(EC 3.2.1.91), and C) β-glucosidases or β-glucoside glucohydrolases (BGL) (EC 3.2.1.21). Endoglucanases cut as random at internal amorphous sites of the cellulose polysaccharide chain, generate oligosaccharides with various lengths and consequently new chain ends. Exoglucanases act as a processive manner on the reducing or non-reducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure (17). The main advantages of using microorganisms for production of CMCase are theirs huge economical production capacity and their easy manipulation to obtain enzymes of desired characteristics (13).

Thermophilic microorganisms are adapted to thrive at temperatures above 50 °C. They are a source of interesting enzymes that are both thermoactive and thermostable (16). The use of microbial enzymes in industrial areas has

been increasing because of its economical production and immobilization of unsolvable materials in water and durable use in respect to biotechnological activities (6). Among the microorganisms, *Bacillus* species are good secretors of extracellular enzymes such as amylase, arabinase, cellulase, lipase, protease, and xylanase which play important roles in many biotechnological processes (2). For applications in industrial processes, the enzymes should be stable at high temperature, pH, presence of salts, solvents, toxicants etc (3).

Because of the importance of using cellulose enzymes in animal feed industry, the aims of this study were isolation, identification of *Bacillus* sp. strain with cellulose activities and improvement of the enzyme production for use in animal nutrition.

MATERIALS AND METHODS

Microorganisms and culture conditions

Bacillus sp. VA1-29 was isolated from coast sediment samples collected from Van Lake, Turkey. To select the Gram-positive spore-forming bacteria *Bacillus* sp., soil sample was incubated at 80 °C for 10 min (7). The isolates were cultivated in LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 9.0) for 24 h at 55 °C with shaking at 200 rpm. The isolates screened for CMCase activity on LB-agar-CMC plates containing (g L-1) tryptone 10, yeast extract 5, NaCl 10, CMC 1 agar 15 (pH 9.0) at 55°C (12). Cellulolytic isolates were selected by flooding the agar plates with Congo-red solution (0.1%)(5).

Enzyme production

The organisms was propagated at 55 °C for 24 h in 100 ml of a LB medium, containing 0.1% CMC, placed in 1000-ml flasks, with shaking on a shaker (200 rpm/min). The initial pH of the medium was about 9.0. After removal of cells by centrifugation (10 000_/g, 20 min) at 4°C, the supernatant was used for partial purification.

Enzyme assay

The relative CMCase activity was assayed by adding 1 ml of enzyme to 1 ml soluble CMC (0.1% w/v) in 50 mM Tris buffer pH 9.0, and incubating at 55 °C for 30 min. The reaction was stopped by the addition of 3 ml of 3, 5-dinitrosalicylic acid reagents. A550nm was measured in a Cecil 5500 spectrophotometer. One unit of amylase activity was defined as the amount of enzyme that released one micromole of reducing sugar equivalent to glucose per minute under the assay condition (16). One enzyme unit is defined as the amount of enzyme releasing 1 mmol of glucose from the substrate in 1 min at 55 °C.

Protein determination

Proteins of wild type and mutant variants were estimated as described by Lowry . (14) using bovine serum albumin as the standard.

Effect of incubation period

The effect of incubation period was determined by assaying the enzyme activity in different incubation periods (12, 24, 36, 48, 60, and 72 h).

Effect of pH and temperature on activity and stability:

Temperature and pH effects on enzyme activity were assayed at various temperatures ranging from 30-100 C and pH values ranging from 6-12 for 30 min. Following buffers were used in the reactions: 100mM Na-phosphate (pH 6-7) and 100 mM Tris (pH7-12) (3).

SDS–PAGE and zymogram analysis

SDS- CMC-PAGE (0.1% CMC) were done as described by laemmli (12) with slab gels (12% w/v acrylamide). For visualizing of total proteins, SDS-PAGE was stained for 1 h with the solution of 0.1% Coomassie blue R250-40% methanol-10% glacial acetic acid and then destained overnight in the same solution without dye. For activity staining (zymogram) of Starch by SDS- Starch-PAGE, SDS was removed by washing the gel at room temperature in solution-A (50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol) for 1 h and solution-B (50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2) for 1 h, respectively. The gel was kept overnight in solution-C (50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), 5 mM β-mercaptoethanol, 1 mM EDTA) at 4°C for renaturation of the enzyme. It was then sealed with film and incubated at 55°C for 4 h. After incubation, the gel was stained in 0.1% (w/v) Congo-red dye for 1 h and washed with 1% (w/v) NaCl for 30 min to visualize the clear band of CMCase activity (13, 4). The molecular mass of the enzyme was finally estimated from the position of standard proteins.

RESULTS AND DISCUSSION

Results

The isolated alkaline, halophilic and thermophilic Bacillus sp. VA1-29 strain from Van Lake in Turkey was gram positive, rod shaped, aerobic, catalase positive and spore forming. According to the basis of various morphological and biochemical characteristic, it was identified as Bacillus sp. Maximum enzyme production was recorded after 12 h at 55 °C. Enzyme synthesis of Bacillus sp. VA1-29 occurred at temperatures between 30 and 100 °C with an optimum of 50 °C. There was a variation in amylase synthesis within the pH range 6.0 and 12.0 with an optimum pH 7.5, while VA1-29 Bacillus sp. grew well at between 7.5 and 8 pH on CMC agar medium in presence of NaCl (5% wt/v) and occurred up to 55 °C.

Enzyme properties

Productions of CMCase at various time courses were investigated. The Bacillus sp. VA1-29 culture was incubated at 55 °C for 12, 24, 36, 48 and 60 hours. Maximum enzyme production was recorded after 12 h at 55 °C (Fig.1).

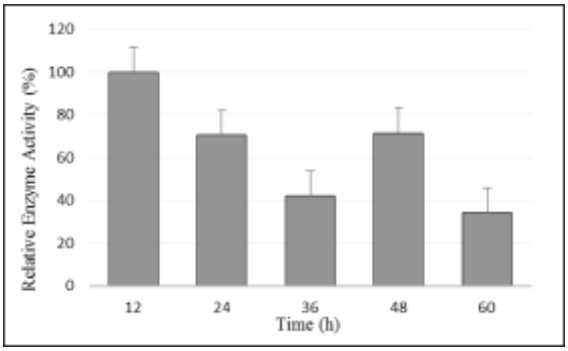

Figure 1. Productions of CMCase at different time by Bacillus sp. isolate VA1-29

The enzyme had a broad temperature range between 30-100 °C and the optimum activity was observed at 50 °C. The relative enzyme activities were 82, 100, 88, 88, 78 and 73 % at 40, 50, 60, 70, 80 and 90, respectively, whereas only 40% activity was retained at 100°C for 30 min (Fig.2). The enzyme also showed a significant relative activity between 7.5 and 8 pH. Effects on enzyme activity was assayed at values ranging from 6-12 for 30 min. Optimum activity was observed at pH 7.5. Following buffers were used in the reactions: 100mM Na-phosphate (pH 6-7) and 100 mM Tris (pH7-12) (Fig. 3).

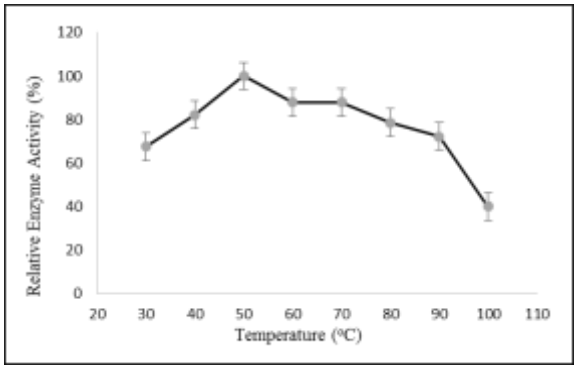

Figure 2. The effect of temperature on CMCase activity

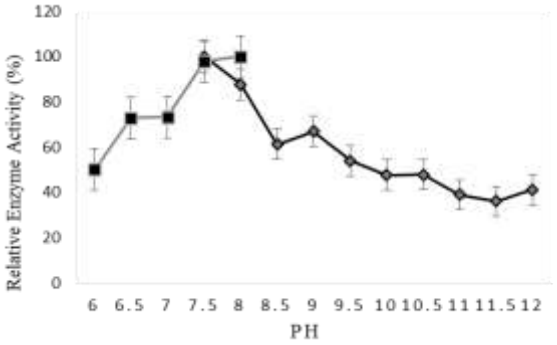

Figure 3. The effect of pH on CMCase activity

Molecular weight

Molecular weights of wild and mutant type of CMCase determined by SDS-CMC-PAGE electrophoresis revealed single bands showing CMCase activity in gel using BioCapt MW software. The molecular mass of bands was 33kDa (Fig. 4).

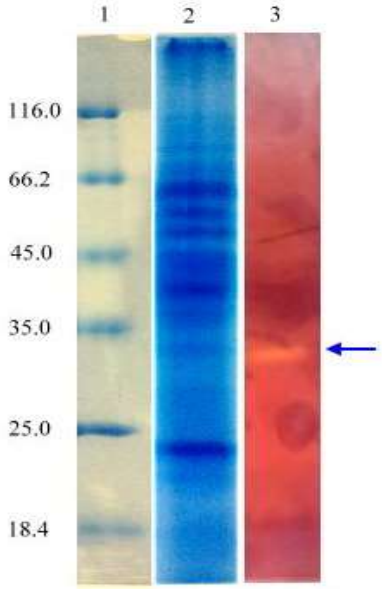

Figure 4. Zymogram analysis of α-amylases on SDS-PAGE. The gel was cut into two pieces, the marker and total proteins were visualized with Coomassiebrillant blue staining and the activity of enzyme revealed by Congo-red (1: Marker, 2: SDS-PAGE, 3: Zymogram)

Specific Activities

Total protein from Isolate Bacillus sp. VA1-29 was analyzed according to the Lowry (1951) method. One mg of protein in a minute was to break 0.6 mmol of substrate.

DISCUSSION

In the present study, water and soil samples were collected from Van Lake of Turkey and used for isolation of Gram (+), spore forming, and aerobic bacterial strains. About 180 strains were isolated and then were screened for CMCase activity. Among these isolates, 24 bacteria showed amylolytic activity on LB-agar plate containing CMC. The Bacillus sp. isolate VA1-29 selected for further studies because of its maximum amylolytic hollow zone around the colony. CMCase from alkaline and thermophilic Bacillus species were previously reported (19, 9, 18, 10, 8, 22, 11).

Most of the Bacillus strains used commercially for the production of CMCase have an optimum pH between 6.0 and 9.0 for growth and enzyme production. The strain Bacillus sp. VA1-29 was improved for CMCase production. Bacillus sp. isolate VA1-29 Maximum enzyme production was recorded after 12 h at 55 °C. The optimum pH values

for native amylases were 7.5. The optimal temperature values for enzyme activity were 50°C. These pH and temperature values are similar to Bacillus Subtilis DLG (19), Pyrococcus horikoshii (10), Bacillus halodurans C-125 (1), Rhodothermus marinus (9), Bacillus sp. HSH-810 (11) enzymes.

Compering the enzymatic properties revealed that the temperature profile of our enzyme had a little difference from other known Bacillus CMCase. Although the optimal temperatures of most bacterial CMCase are in the range of 50-90 ºC, the activity of enzyme is significantly decreased at temperatures lower than 50 ºC and upper 90 ºC. As well as the optimal pH of most bacterial CMCase are in the range of 7-10. It was reported that the broad range of temperatures and the enzyme's high activity at both moderate and upper temperature values make enzymes highly attractive for both basic research studies and industrial processes (15).

The molecular weight of CMCase was 33 kDa on SDS-PAGE. Similar findings between 28 kDa and 50 kDa have been reported previously (8, 20, 9). These differences of molecular weights of CMCase depend on the genes from the organisms.

CONCLUSION

In this study, we isolated the *bacillus sp*. VA1-29 strain produced of thermostable α-amylases. The VA1-29 α-amylases is the thermostable (50 ºC) and slightly alkaline with wide range of pH (7.5-8). Hence, it is qualified for use in biotechnological applications and all its properties make it a useful tool for feed additive in animal and poultry nutrition. The VA1-29 CMCase production process can be commercialized in food animal industry after further optimization and fermentation used as feed additive in animal nutrition.

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