Pullulanases of alkaline and broad pH range from a newly isolated alkalophilic Bacillus sp. S-1 and a Micrococcus sp. Y-1

Cheorl-Ho Kim1, Ho-Ill Choi2 and Dae-Sil Lee3

Genome Research Program1,2 and Laboratory of Molecular Biology3, Genetic Engineering Research Institute, KIST, Taedok Science Town, Yusung-ku, Taejon, Korea.

(Received 27 July 1992; revised 3 January 1993; accepted 18 January 1993)

Key words: Alkalophilic bacteria; Alkaline pullulanase; Multifunctional pullulanase; (Bacillus sp. S-1); (Micrococcus sp. Y-1)

SUMMARY

Two highly alkalophilic bacteria, and potent producers of alkaline pullulanase, were isolated from Korean soils. The two isolates, identified as Bacillus sp. S-1 and Micrococcus sp. Y-1, grow on starch under alkaline conditions and effectively secrete extracellular pullulanases. The two isolates were extremely alkalophilic since bacterial growth and enzyme production occurred at pH values ranging from pH 6.0 to 12.0 for Micrococcus sp. Y-1 and pH 6.0 to 10.0 for Bacillus sp. S-1. Both strains secrete enzymes that possess amylolytic and pullulanolytic activities. Extracellular crude enzymes of both isolates gave maltotriose as the major product formed from soluble starch and pullulan hydrolysis. Compared to other alkalophilic microbes such as Micrococcus sp. (0.57 units ml-1), Bacillus sp. KSM-1876 (0.56 units ml-1) and Bacillus No. 202-1 (1.89 units ml-1), these isolates secreted extremely high concentrations (7.0 units ml-1 for Bacillus sp. S-1 and 7.6 units ml-1 for Micrococcus sp. Y-1) of pullulanases in batch culture. The pullulanase activities from both strains were mostly found in the culture medium (85-90%). The extracellular enzymes of both bacteria were alkalophilic and moderately thermoactive; optimal activity was detected at pH 8.0-10.0 and between 50 and 60 °C. Even at pH 12.0, 65% of original Y-1 pullulanase activity and 10% of S-1 pullulanase activity remained. The two newly isolated strains had broad pH ranges and moderate thermostability for their enzyme activities. These results strongly indicate that these new bacterial isolates have potential as producers of pullulanases for use in the starch industry.

INTRODUCTION

Debranching enzymes hydrolyze the α-1,6-glucosidic linkages of oligo- and polysaccharides such as pullulan, glycogen, amylopectin and β-limited dextrin. Enzymes such as pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41), isoamylase (glycogen 6-glucanohydrolase, EC 3.2.1.68) and amylo-1,6-glucosidase (amylo-1,6-glucosidase/1,4-α-glucan: 1,4-α-glucan 4-α-glycosyltransferase, EC 3.2.1.33) are well known [14]. The most important industrial application of these enzymes is for production of glucose or maltose, when used in combination with glucoamylase or β-amylase, respectively. Recently, 6-O-a-maltosyl- and 6-O-a-maltotriosyl cyclodextrins are being produced in high yields by applying the condensation reaction or transfer reaction of the enzymes [17].

Pullulanase hydrolyses the α-1,6-glucosidic linkages in pullulan and starch [14]. Well-known producers are Klebsiella pneumoniae [24], Bacillus acidopullulyticus [29] and B. flavocaldrius [33]. Pullulanases from K. pneumoniae and B. acidopullulyticus are used for saccharifying starch to produce glucose and maltose on an industrial scale [14]. Over the last decade, a variety of pullulanolytic enzymes with different substrate specificities have been characterized [31]. The enzymatic classification of pullulan-degrading enzymes has four groups based on substrate specificity and products [23]: (1) pullulan hydrolase type I is the enzyme that attacks α-1,4-glycosidic linkages in pullulan forming panose (it was previously classified as neopullulanase [18]); (2) pullulan hydrolase type II attacks α-1,4-glycosidic linkages in pullulan forming isopanose (it was previously classified as isopullulanase [28]); (3) pullulanase type I specifically hydrolyses the α-1,6-linkages in pullulan forming maltotriose; (4) pullulanase type II attacks, in addition to α-1,6-linkages in pullulan, α-1,4-linkages in other polysaccharides.

Recently, some thermostable pullulanases have been studied from extreme thermophilic microorganisms [8,9,16,23]. However, alkalophilic microorganisms, producing extracellular alkaline pullulanase, have been reported in only three studies [2,13,21]. For industrial application of pullulanase, it is desirable that enzymes have activity over alkaline and a broad pH range. We describe the isolation of two alkalophilic bacteria producing alkaline and broad pH range pullulanases.
MATERIALS AND METHODS

Media and cultivation

Pure cultures were obtained by repeated serial dilution with a alkaline Basal Medium (10-fold stepwise). The bacteria were grown on alkaline Basal Medium composed of (w/v) 1% soluble starch (from Wako Pure Chemical Co. Ltd, Tokyo, Japan), 1% pullulan (M, about 65,000; Hayashibara Biochemical Lab., Okayama, Japan) and 1% dextran (M, about 200,000; Sigma Co., St Louis, MO, USA), 0.5% Bacto peptone (Difco, Detroit, MI, USA), 0.1% Bacto yeast extract (Difco), 0.1% K2HPO4, 0.1% NaCl, 0.1% MnCl2, 0.2% MgSO4·7H2O, 1% Na2CO3, 1 ml (v/v) trace element solution. The trace element solution comprised (mg per litre of distilled water): FeSO4·7H2O, 1000; H3BO3, 300; CoCl2·6H2O, 190; ZnCl2, 42; NiCl2·6H2O, 24; NaMoO4·2H2O, 18. Na2CO3 was separately added to the basal medium after autoclaving. Media were solidified by the addition of 2.0% (w/v) Bacto agar (Difco) for plates.

For large-scale production of pullulanases, bacteria were cultivated aerobically at 50 °C and pH 8.0 (for S-1 strain) and pH 10.0 (for Y-1 strain) in a 5-litre stirred-tank fermentor (Korea Fermentator Co. Ltd, Seoul, Korea) for 36 h. The liquid medium for enzyme production contained 1% soluble starch instead of pullulan.

Isolation of alkaline bacteria producing pullulanase

Yusung Hot Spring area is a large hot-spring region located near Taean in the central area of Korea. The sediment soil samples (0.5 g) for screening were obtained from hot-spring sources in Yusung hot-spring park. The samples were suspended in 10 ml of sterile water and spread onto soluble starch, dextran, or pullulan-reactive red agar plates [2], and incubated at 50 °C for 3 days. Colonies that had formed a clear zone around their margins were picked, inoculated into a liquid medium composed of (w/v) 1% soluble starch at 50 °C and pH 8.0 (for S-1 strain) and pH 10.0 (for Y-1 strain) in a 5-litre stirred-tank fermentor (Korea Fermentator Co. Ltd, Seoul, Korea) for 2 days. The pullulan-reactive red agar containing 1% soluble starch instead of pullulan.

Enzymatic assays

Pullulanase and amylase activities were measured by determining the reducing sugar released from pullulan and soluble starch, respectively. The reaction mixture for each assay was the same as that described previously [10]. Enzymatic activities of amylase and pullulanase were measured in 50 mM glycine-NaOH buffer, pH 9.0 or 10.0, at 50 °C. Suitably diluted enzyme (50 μl) was added to 150 μl substrate and incubated for 30 min. The reducing sugar liberated was quantified by following the 3,5-dinitrosalicylic acid (Merck, Darmstadt, FRG) method [20]. One unit of each enzyme activity was defined as the amount of enzyme which released 1 μmol of reducing sugar, expressed as glucose per min under the reaction conditions.

Activity staining of S-1 and Y-1 pullulanase

Activity staining of pullulanase in the slab gel was done essentially by the method of Ara et al. [2] with pullulan-reactive red agar plates as replica plates. The slab gel after