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Isolation and Screening of Chitosanase Producing Microorganisms

Sutee Wangtueai[1], Wanchai Worawattanamateekul[1]*,
Mathana Sangjindavong[1],Nuanphan Naranong[2] and Sarote Sirisansaneeyakul[3]

ABSTRACT

A total of 183 isolates of microorganisms were collected from forest soil with the shrimp wastes deposited. The selected four bacterial isolates with high activity of chitosanolytic enzymes on chitosanase detected agar plates (CDA-plates) were TP11.03, TP11.22, TP12.24 and KU22.13. Among them, the potential isolate TP12.24 was identified as *Bacillus cereus* which produced the highest amount of chitosanase on M9-Chitosan medium.

Key words: chitosan, chitosanase, *Bacillus cereus*, isolation

INTRODUCTION

Chitosan, a chitin deacetylated polymer has been widely used in agriculture, food, medical, pharmaceutical, cosmetic, paper and textile (Knorr, 1984; 1991). It is a potential substrate which could be chemically or microbially converted to the valuable chitosanoligosaccharides (Jeon and Kim, 1998; Honda *et al*., 1999; Choi *et al*., 2002; 2000; 2001). Although chitosano-oligosaccharides play essential role in wide-ranged applications, their conversion is economically limited during to the limited sources of chitosanase.

Chitosanase, the active enzymes hydrolyzing chitosan, can be produced from several microorganisms including fungi, bacteria and actinomycetes which are naturally occurring in the rhizosphere environment. The purified chitosanase has been previously used in the preparation of the chitosanoligosaccharides from chitosan. These pronounced oligosaccharides have immunopotentiating properties (Suzuki *et al.*, 1984; Papineau *et al.*, 1991), antimicrobial activities (Price and Storck, 1975; Tominaga and Tsujisaka, 1975; Somashekar and Joseph, 1996; Jeon *et al*., 2001; No *et al*., 2001) and antitumor activities (Jeon and Kim, 1998; 2000).

The purpose of this present study was to isolate and screen the chitosanase-producing microorganisms from soils.

MATERIALS AND METHODS

Soil sample preparation

Raw shrimp heads and raw shrimp shells were deposited on forest soils up to 1 foot and allowed to decay. The galvanized iron sheets were used to cover the holes to protect the samples against animals. The deposition of shrimp wastes was done to attract possible chitosanase-producing microorganisms present in the soils and increased

1 Department of Fishery Products, Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand.
2 Department of Applied Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.
3 Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail:

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the success rate of isolation. After two weeks, soil samples were collected and brought to the laboratory for microbial isolation.

Isolation of chitosanase producing microorganisms

One gram of soil samples was added to sterile water with suitable serial dilution. The spread plating method was used to isolate the soil microorganisms. From each dilution, 0.1 ml was transferred to the Petri plates containing the chitosanase-detection agar medium (CDA) (Cheng and Li, 2000). The plates were incubated at 30°C and observed after 3 days incubation for colony formation. Total count of viable cells and viable cells forming clear zone around the colonies were conducted and those colonies with clear zone were collected for further purification.

Screening of chitosanase producing microorganisms

The first screening procedure of the isolates was done by point inoculation of pure cultures on the CDA-agar plate which were incubated at 30°C for 3 days. The diameters of colony and clear zone around colonies were determined to select the high microbial activity of chitosanolytic enzymes.

The second screening procedure of the high chitosanolytic enzymes producing microorganisms was carried out in liquid medium. Microbial cells were inoculated into the 500-ml Erlenmeyer flasks containing 200 ml of M9-Chitosan medium with 1 % chitosan and incubated at 30°C for 96 h on a rotary shaker at 250 rpm. The samples were taken every 6 h to determine the total viable cell count, cell dry weight and enzyme activity.

Strain identification

The API 50CH strip was used to identify the bacterial isolate which performed at Microbiological Resources Centre, Thailand Institute of scientific and Technology Research (TISTR).

RESULTS AND DISCUSION

Isolation of chitosanase producing microorganisms

After raw shrimp wastes were deposited on a forest soil at Thayang district, Petchaburi province and on the soil at Kasetsart University, Thailand for two weeks, the soil samples were taken to isolate the chitosanolytic enzymes producing microorganisms. The total count of viable cells from 3.3×10^4 to 7.2×10^5 CFU/g and the total count of viable cells forming clear zone around the colony accounted from 3.0×10^3 to 4.1×10^5 CFU/g. The ratio of total viable cells to the cells that formied clear zone around the colonies ranged from 1.5 to 11.0 (Table 1). Among them, total 183 colonies of soil contaminated microorganisms were isolated and used to screen for the high chitosanolytic enzymes producing strains.

Screening of chitosanase-producing microorganisms

The screening of 183 isolates was done by point inoculation on CDA plate. It was observed that the isolates formed clear zone around colonies on CDA plate after 3 days cultivation. The numbers of isolated cultures which were 58, 30, 28, 29, 27 and 5 had the clear zone diameter ranges 0-1.0, 1.1-2.0, 2.1-3.0, 3.1-....., respectively. The 61 isolates showing the high diameter ratio between 3.1-6.0 were further investigated for their morphology and Gram staining. The 46 bacterial isolates were found to be Gram-positive and the rest of them were Gram-negative bacteria. Most of them were rod-shape bacteria under the light microscopic observation. Thus the highest diameter ratio of 4 isolates (4.91-5.33) were selected to determine the production of extracellular chitosanolytic activity in the M9-chitosan medium of these four isolates, TP11.03 (5.25), TP11.22 (5.20) and TP12.24 (5.33) were Gram-positive rod shape, while KU22.13 (4.91) was the only gram-negative short rod.

Table 1 Total viable count of cells, clear zone forming and ratio from shrimp wastes contaminated soils at 4 different places.

Soil samples	Total viable cells (CFU/g)	Total viable cells forming clear zone (CFU/g)	The ratio of total viable cells to the total viable cell forming clear zone	Number of isolates	Isolates no.
I	2.3×10^5	1.5×10^5	1.5	36	TP11.01-TP11.36
II	7.2×10^5	4.1×10^5	1.7	25	TP12.01-TP12.25
III	6.2×10^4	3.2×10^4	1.9	34	TP21.01-TP21.34
IV	1.2×10^5	4.0×10^4	3.0	17	TP22.01-TP22.17
V	3.5×10^4	1.5×10^4	2.3	8	KU11.01-KU11.08
VI	3.3×10^4	3.0×10^3	11.0	19	KU12.01-KU12.19
VII	9.5×10^4	9.0×10^3	10.5	22	KU21.01-KU21.22
VIII	4.9×10^4	4.5×10^3	10.8	22	KU22.01-KU22.22

Note: I-IV were from the mountain soils at Thayang district, Petchaburi province, V-VII were from the campus soils at Kasetsart University.

Production of chitosanase by the selected isolates

The four isolates were cultured in M9-chitosan medium and it was found that they had difference in growth. Nevertheless, there was no significant change in pH of the culture broth (pH 6.0-6.2) in liquid cultivation. The highest extracellular chitosanolytic activities produced by the isolates TP11.03, TP11.22, TP12.24 and KU22.13 were 638.86 U/l in 24 h, 1,542.50 U/l in 48 h, 1,943.89 U/l in 66 h and 1,483.66 U/l in 66 h, respectively. These results corresponded to the solid cultivation showing the high diameter ratio. The liquid cultivation yielded a very useful kinetic parameter for the screening of high chitosanolytic enzymes producing microorganisms. This is similar to the reports of Carroad and Tom (1978) and Yoshihara *et al.* (1990). The kinetic parameters of cell growth and chitosanase production are summarized in Table 2. The bacterial isolate TP11.22 showed highest specific growth rate and volumetric enzyme productivity, while isolate TP11.24 gave the maximal specific rate of enzyme production and final enzyme activity. Therefore, the lather was finally selected as a potential chitosanase producing bacteria. The strain was further identified systematically and used as the model microorganism for studying the production of chitosanase in submerged batch fermentation.

Strain identification

After biochemical testing using API 50CH strip which was performed by TISTR, the newly isolated TP12.24 was identified as *Bacillus cereus*, as morphologically shown in Figure 1. The biochemical characteristics of the strain are summarized in Table 3.

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Table 2 The fermentation kinetics for chitinolytic enzymes production of four selected isolates.

Isolates	Specific growth rate (μ, h^{-1})	Specific rate of enzyme production (q_p, U/g cell h)	Volumetric rate of enzyme production (Q_p, U/l h)
TP11.03	0.175	156.83	20.31
TP11.22	0.188	75.63	31.69
TP12.24	0.182	224.36	24.68
KU22.13	0.125	148.68	20.89

Note: m obtained from plotting graph between log cell dry weight and culture time, while q_p and Q_p taken from the calculation.

Table 3 Biochemical characteristics of the TP12.24 strain identified as *Bacillus cereus* after TISTR.

Gram reaction	+ve		
Fermentative production of acid from	Reaction	Fermentative production of acid from	Reaction
Glycerol	+	Salicine	+
Erythritol	-	Cellobiose	+
D-arabinose	-	Maltose	+
L-arabinose	-	Lactose	-
Ribose	+	Melibiose	-
D-xylose	-	Sucrose	+
L-xylose	-	Trehalose	+
Adonitol	-	Inulin	-
β-methyl-D-xyloside	-	Melezitose	-
Galactose	-	D-raffinose	-
D-glucose	+	Starch	+
D-fructose	+	Glycogene	+
D-mannose	-	Xylitol	-
L-sorbose	-	β-gentiobiose	+
Rhamnose	-	D-turanose	-
Dulcitol	-	D-lyxose	-
Inositol	-	D-tagatose	-
Mannitol	-	D-fucose	-
Sorbitol	-	L-fucose	-
α-methyl-D-mannoside	-	D-arabitol	-
α-methyl-D-glucoside	-	L-arabitol	-
N-acetyl-glucosamine	+	Gluconate	+
Amygdaline	+	2-keto-gluconate	-
Arbutine	+	5-keto-gluconate	-
Esculine	+		

Note: +ve = Gram positive bacteria, + = Positive reaction, - = Negative reaction

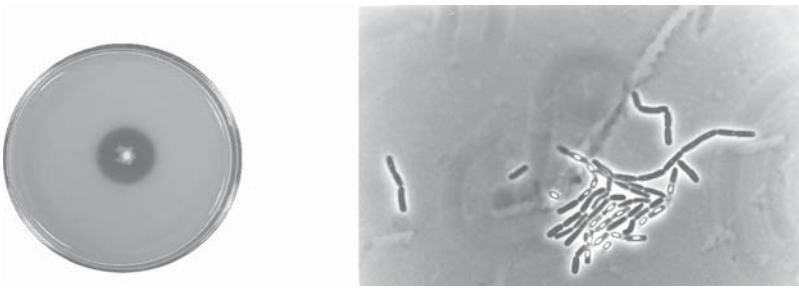

Figure 1 Characteristic of clear zone around colony on CDA-plate (left) and light microscopic observation (right) of the new isolate *Bacillus cereus* TP12.24.

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