CLONING OF PULLULANASE GENE FROM LOCAL ISOLATED BACTERIA

Chee Chit Lai[1], Rosli Md. Illias[1,*], Osman Hassan[2], Kamarulzaman Kamaruddin[3], Suraini Abdul Aziz[4], Madihah Md. Salleh[5], Wan Salwanis Wan Md Zain[1].

[1]Department of Bioprocess Engineering, Faculty of Chemical Engineering and Natural Resources Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia. [2]School of Chemical and Sciences, Faculty of Science and Technology, Universiti Kabangsaan Malaysia, 43000 Bangi, Selangor. [3]Bioprocess and Chemical Technology Centre, SIRIM Berhad, Malaysia. [4] Department of Biotechnology, Faculty of Food Science and Biotechnology, Universiti Putra Malaysia, 43400 Serdang, Malaysia. [5]Department of Biology, Faculty of Science, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia.

Pullulanases (pullulan-6-glucanohydrolase, EC 3.2.1.41) are debranching enzymes that are able to hydrolyze α-1, 6-glycosidic linkages in pullulan and branched polysaccharides, producing maltotriose. Pullulanases are a member of family 13 glycosyl hydrolases or α-amylase family. Pullulanase are classified in two categories based on substrate specificity: Type I pullulanases that only hydrolyze α-1,6 linkages, and Type II pullulanases that hydrolyze α-1,6 and α-1,4 linkages. Due to this debranching ability, pullulanases are used in combination with α-glucosidase to improve saccharification rate and yield. In this project, local isolated bacteria were screened for the pullulanase activity by using AZCL-pullulan and Red pullulan. Bacteria S7 and bacteria P2 exhibited the ability to degrade AZCL-pullulan. However, Red pullulan plate growth with bacteria P2 exhibited halo zone. This result confirmed that bacteria P2, which was isolated from local resources, is a pullulanase-producer. 1kb pullulanase gene fragment was amplified using PCR. Four conserved regions of amylolytic enzyme and a highly conserved region of pullulanase type I (YNWGYDP) were identified within the deduced amino acid sequence. Bacteria P2 was identified as *Exiguobacterium sp.* MAA-1 using 16S rRNA gene sequence analysis.

* *Corresponding author. Email: r_rosli@utm.my*

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INTRODUCTION

Pullulanases (pullulan-6-glucanohydrolase, EC 3.2.1.41) are debranching enzymes that are able to hydrolyze α-1, 6-glycosidic linkages in pullulan and branched polysaccharides, producing maltotriose. Pullulan, which is a linear polymer of maltotriose linked by α-1,6-glycosidic linkages, serves as a model substrate for pullulanase. Pullulanases are a member of family 13 glycosyl hydrolases or α-amylase family. Pullulanase are classified in two categories based on substrate specificity: Type I pullulanases that are only hydrolyze α-1,6 linkages, and Type II pullulanases that hydrolyze α-1,6 and α-1,4 linkages. Due to this debranching ability, pullulanases are used in combination with α-glucosidase to improve saccharification rate and yield. This study was carried out to identify locally isolated pullulanase-producer and to isolate the pullulanase gene from the bacteria.

MATERIALS & METHODS

Modified Peptone-Yeast Extract (PYE) Medium (g/L)

0.1% (w/v) NH_4Cl, 0.267% $Na_2HPO_4.12H_2O$, 0.03% KH_2PO_4, 0.3% yeast extract, 1.0% peptone, 0.02% $MgCl_2.6H_2O$, 2.0% starch and 2.0% agar (optional).

AZCL-Pullulan Agar (g/L)

AZCl-pullulan agar was prepared as two layers. Bottom layer: 1.0% peptone, 0.1% NH_4Cl and 1.5% agar. Upper layer: 1.0% peptone, 0.1% NH_4Cl, 0.01-0.02% AZCl-pullulan and 1.0% agar.

Red-pullulan Agar (g/L)

1% peptone, 0.1% NH_4Cl, 0.5% red pullulan (Megazyme), and 2% agar

Bacteria

Bacteria 9, 2, 14, 90, 39, NA, 26, 89, 7, 89, 8, 23, 19, 1, 90, 48, P2, P3, & S7 were screened by using AZCl-pullulan plate and red-pullulan plate. All the bacteria were incubated at 50°C, except bacteria P2 and P3, which were incubated at 37°C, and bacteria S7 that was incubated at 45°C.

DNA Manipulation and Cloning Procedures

Genomic DNA of bacteria P2 was isolated according to the Ish-Horowitz method (1981). Plasmid DNA isolation was carried out using Alkaline Lysis Method. The rest of the DNA manipulations were performed according to the manufacturer's manual.

PCR Amplification

Degenerated oligonucleotides were used to amplify part of the pullulanase gene. The forward primer Mix1 is 5'- TAT AAT TGG GGD TAT GAT CC -3' while the reverse primer ReMix1 is 5'- CCT AGT ATD GGG GTT AAT AT-3'. The 16S rRNA gene of bacteria P2 was amplified using universal primers of eubacterial 16S rRNA. The forward primer Forward-B27f is 5'- AGA GTT TGA TCC TGG CTC AG –3' and the reverse Reverse-U1492r is 5'- GGT TAC CTT GTT ACG ACT T –3'.

Nucleotide Sequence Analysis

The nucleotide sequence of the PCR product and the deduced amino acid sequence were compared with existing sequences using BLASTN and BLASTX program provided by National Center for Biotechnology Information (NCBI). The sequences were analyzed using DNASIS MAX 1.0 software (Hitachi Software Engineering Co., Ltd.).

RESULTS & DISCUSSION

One of the reliable methods to screen for pullulanase-producer is through the degradation of dyed-pullulan. From all the bacteria that were screened, only two strains of bacteria, bacteria S7 and bacteria P2, exhibited the ability of AZCL-pullulan degradation. However, only bacteria P2 exhibited halo zone on Red-pullulan plate. Figure 1 shows the degradation of dyed-pullulan by bacteria P2.

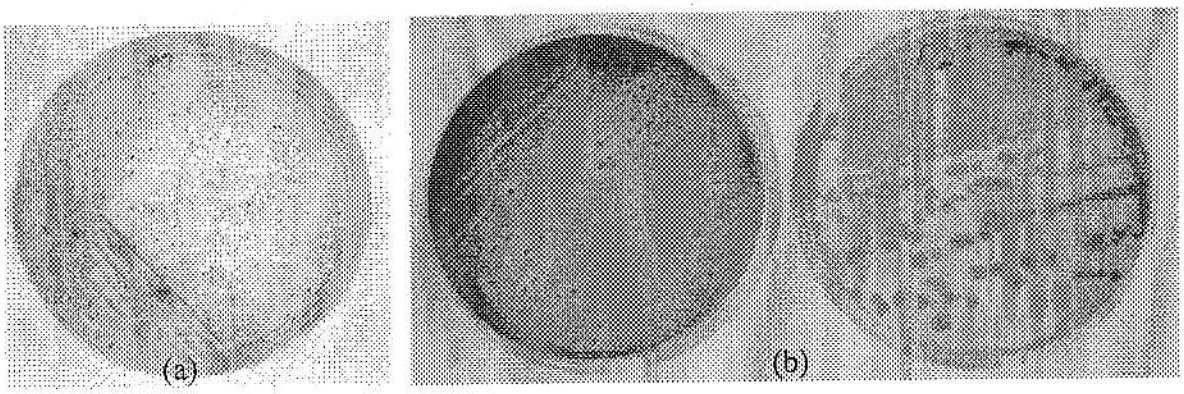

Figure 1: (a) Degradation of AZCl-pullulan by bacteria P2. (b) Degradation of red pullulan agar by bacteria P2. [Left: After 1 day incubation at 37°C, right: after two days incubation at 37°C].

A 1kb pullulanase gene fragment of bacteria P2 was amplified using PCR method. The deduced amino acids sequence of the gene fragment showed a highest homology of 60% with pullulanase gene from *Exiguobacterium sp.* 255-1. Based on the multiple alignment, the sequence of the amplified product contains all four conserved regions of amylolytic enzyme, conserved region I, II, III & IV, and a highly conserved region of pullulanase type I (motif YNWGYDP) (Table 1). This proved that the PCR amplified fragment is a essential part of pullulanase gene as the YNWGYNP motif is found only in type I pullulanase gene. This motif is believed to be involved in the degradation of α-1, 6-glycosidic linkages. The four conserved regions are found in all amylolytic enzymes, and it was proposed to be involved in substrate binding.

Table 1: Regions conserved among pullulanases from different microorganism and comparison with amplified product mix1. [The underlined amino acid residues are those identified by Nakajima et al. as conserved among all amylolytic enzymes (Bertoldo et al., 1999)].

Source	Starting position and amino acid sequence for conserved region				
	YNWGYDP	I	II	III	IV
F. pennavorans Ven5	424 YNWGYDP	465 GIRVILDMVFPHT	538 DGFRFDQMGL	570 YGEPWGG	648 PQETINYVEVHDNHTLWD
T. maritime	430 YNWGYDP	473 FTGVIMDMVFPHT	548 DGFRFDQMGL	580 YGEPWGG	638 PEETINYAACHDNHTLWD
B. thetaiotaomicron	234 YNWGYDP	275 GIRVIMDVVYNHT	347 DGFRFDLMGI	379 YGEGWAA	670 PVQMISYVSCHDGLCLVD
Thermus sp.	572 YNWGYDP	333 GLRVVMDAVYNHV	405 DGFRFDLMGV	437 YGQGWDL	512 PRQSINYVECHDNHTFWD
B. acidopullulyticus	436 YNWGYDP	476 RIAINMDVVYNHT	549 DGFRFDLMAL	580 YGEPWTG	656 PSETINYVTSHDNMTLWD
C. saccharolyticus	405 YNWGYDP	445 GIGVVMDVVFNHT	520 DGFRFDLMGL	552 YGEGWVM	629 PDECVNYVSCHDNLTLFD
K. aerogenes	574 YNWGYDP	615 GMNVIMDVVYNHT	691 DGFRFDLMGY	723 FGEGWDS	842 PTEVVNYVSKHDNQTLWD
K. pneumoniae	562 YNWGYDP	603 GMNVIMDVVYNHT	679 DGFRFDLMGY	711 FGEGWDS	830 PTEVVNYVSKHDNQTLWD
B. sterothermophilus	572 YNWGYDP	613 GMNVIMDVVYNHT	689 DGFRFDLMGY	721 FGEGWDS	856 PTEVVNYVSKHDNQTLWD
Bacteria P2	**YNWGYDP**	**GIRVIMDVVFNHT**	**DGFRFDLMGL**	**IGEGWDL**	**PNQVVNYVECHDNLTLWD**

Bacteria P2 was also identified at 16s rRNA level. The 1.5kb 16S rRNA gene fragment was successfully amplified from bacteria P2 using universal primers. The result show that the sequence share very high identities to genus *Exiguobacterium*, reaching up to 98%. Bacteria P2 was identified as *Exiguobacterium sp.* MAA-1.

CONCLUSION

Bacteria P2 showed the ability of degradation both AZCL-pullulan and red-pullulan. This confirmed that bacteria P2, which isolated from local resources, is a pullulanase-producer. Nearly 1kb of sequence was amplified and proved as a part of pullulanase gene containing four conserved regions of amylolytic enzyme (conserved region I, II, III & IV) and a highly conserved region of pullulanase type I (motif YNWGYDP). Around 1.5kb 16S rRNA gene nucleotide sequence share very high identity of 98% to genus *Exiguobacterium*. Bacteria P2 was identified as a *Exiguobacterium sp.* MAA-1.

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