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Primer Design of the Cellulase Gene from *Bacillus cereus*

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Abstract: *Cellulase is an enzyme that can be produced by fungi, bacteria, and protozoa that can decompose cellulose and some related polysaccharides. Cellulase is an enzyme that breaks β-1,4 glycosidic bonds in cellulose. Cellulase has the greatest demand because of its various industrial applications such as food, textile, paper, detergent industries, and pharmaceuticals industries. Detection of the cellulase gene on the PCR instrument requires a primer. This research was aim to obtain the best pair of primers for the cellulase gene from Bacillus cereus designed in silico using the application of NCBI*
Materials and Methods*: The cellulase gene of Bacillus cereus was obtained from the data available on the website http://www.ncbi.nlm.nih.gov (NCBI). Then search for primary candidates with certain criteria using the same site.*
Results*: A primer candidate has been selected from no 6 with forward primer ATGAACCACATCCAGAAACA and reverse primer TCTTTGTCCACTTCACGAAT. With the length of each primer (forward and reverse) was 20 nucleotides. The Tm in this primer were 54.84 ^{o}C and 54.92 ^{o}C, respectively. GC percent for forward and Reverse were 40%. This selected primer has 1 GC clamp on the forward and 2 GC clamp on the reverse.*
Key Word*: Bacillus cereus ; Cellulase; Gene; PCR; Primer.*

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I. Introduction

Cellulase is an enzyme that breaks β-1,4 glycosidic bonds in cellulose[1]. The use of cellulose for the pharmaceutical sector includes as a tablet filler for the direct compression method, as a binder, disintegrating tablets, accelerating drug delivery systems[2].

Molecular biology techniques such as polymerase chain reaction (PCR) can be used to detect the presence of genes (genetic material) consisting of a piece of DNA that determines individual traits. PCR is a tool that can be used to duplicate the target gene[3]. Primer is needed when duplicating the target gene. Primary molecules can be DNA, RNA, or even specific proteins. Primer is used as a barrier for target DNA fragments to be amplified and at the same time provides a hydroxy group at the 3' end which is needed for the DNA extension process. Both of these primers must be present in the polymerization reaction for DNA amplification to occur. Primary consists of forward and reverse.

Amplification DNA in PCR consists of three stages. The initial stage of the amplification process is denaturation of the DNA strand where the double-stranded DNA molecule is converted into a single strand, then the primer pair attaches to the target DNA fragment (annealing). The last stage is the process of extending the DNA sequence with the help of DNA polymerase[4]. Characteristics that should be considered when designing primer :

a. Primary length
b. Percentage of GC content
c. Melting temperature
d. Hairpin
e. self dimer

The primer design in this study was carried out by in silico experiments to make the cellulase gene amplification process by PCR more efficient and reduce the cost of using primers. This research is a follow-up study from[5] that has successfully screened cellulase enzymes from *Bacillus cereus* isolates taken from cow manure compost samples. The aim of research was to obtain the best pair of primers from the cellulase gene designed in silico using the application of NCBI

II. Material And Methods

Cellulase Encoding Gene

The gene encoding the cellulase from *Bacillus cereus* was obtained from a search using NCBI (the National Center for Biotechnology Information) with keywords *Bacillus cereus* and cellulase.

Primer design

The *Bacillus cereus* nucleotide sequence encoding the cellulase gene obtained from NCBI was used to design the primers. Primer design using the online application through NCBI Web Server by clicking Analyze-Primer-BLAST- Pick Primer. The PCR product size and primer melting temperature were adjusted. NetPrimer is used to see the primary characteristics. Analysis of candidate primer pairs was performed (melting temperature, %GC, length of PCR product, hairpin structure, cross dimer).

III. Result

The search for nucleotide sequences of the cellulase gene from *Bacillus cereus* was carried out using NCBI website with the keywords cellulase and *Bacillus cereus* in the nucleotide box. Sequence data were obtained with the gene bank code KC503888.1, namely *Bacillus cereus* strain Y5 bacteria that can produce cellulase, and was used as a template as shown in Figure 1 below.

Bacillus cereus strain Y5 cellulase gene, complete cds

GenBank: KC503888.1

FASTA Graphics

Go to:

```
LOCUS       KC503888               1269 bp    DNA     linear   BCT 28-APR-2013
DEFINITION  Bacillus cereus strain Y5 cellulase gene, complete cds.
ACCESSION   KC503888
VERSION     KC503888.1
KEYWORDS    .
SOURCE      Bacillus cereus
  ORGANISM  Bacillus cereus
```

Figure 1. *Bacillus cereus* encoding the cellulase gene

The size of the PCR product was set in the range of 100-500 bases for 10 primer pairs. The primary melting temperature is set at an optimum temperature of 55°C, a minimum temperature of 50°C, and a maximum temperature of 60°C. After obtaining the nucleotide sequence of Bacillus cereus that encodes cellulase, the primary design was carried out using an application from the NCBI web. Similar sequences appear after running to obtain primers from the template. From these sequences, it was traced to Bacillus cereus which had the cellulase gene as the target gene. From the web obtained 9 pairs of primary candidates that match the desired target. The 9 pairs of primary candidates can be seen in Figure 2 below.

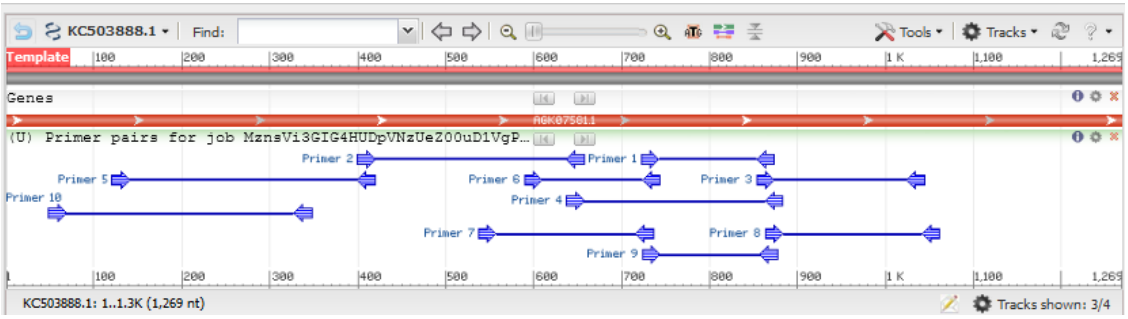

Figure 2. Primer map

From the nine pairs of the primer candidates, all of them had the same primer length of 20 nucleotides with an amplicon length ranging between 151-299 bp (Table 1).

Primer pair		Sequence (5'->3')	Primer length	Amplicon	Start	Stop
1	F	CATTCGTGAAGTGGACAAAG	20	151	723	742
	R	ACCTTGGCTCGTTAATTCAT	20		854	873
2	F	TCACAAGGAATGAAAGTCGT	20	258	400	419
	R	TTTCTCGTAATCTTCCGTCC	20		638	657
3	F	ATGAATTAACGAGCCAAGGT	20	191	854	873
	R	AATTCCAAACTCCTCTGCAA	20		1025	1044
4	F	GGACGGAAGATTACGAGAAA	20	246	638	657
	R	TTTGTTTTTCACCTTGGCTC	20		864	883
5	F	CACAAGGGCGATTCAAAAAT	20	299	121	140
	R	ACGACTTTCATTCCTTGTGA	20		400	419
6	F	ATGAACCACATCCAGAAACA	20	154	590	609
	R	TCTTTGTCCACTTCACGAAT	20		724	743
7	F	CTCGCTCTGGAACTAAAAGA	20	199	538	557
	R	CCACTTCACGAATGGAGTTA	20		717	736
8	F	GAGCCAAGGTGAAAAACAAA	20	198	864	883
	R	CCTGGAACAGTACGGTTAAT	20		1042	1061
9	F	ATTCGTGAAGTGGACAAAGA	20	154	724	743
	R	TTTCACCTTGGCTCGTTAAT	20		858	877

Table 1. Primer candidate

Melting temperature and %GC data were also obtained from the nine pairs of primers as shown in Table 2

Primer pair		Tm (^{o}C)	GC (%)
1	F	54.94	45.00
	R	55.03	40.00
2	F	54.92	40.00
	R	54.93	45.00
3	F	55.03	40.00
	R	55.14	40.00
4	F	54.93	45.00
	R	54.89	40.00
5	F	55.13	40.00
	R	54.92	40.00
6	F	54.84	40.00
	R	54.92	40.00
7	F	54.84	45.00
	R	55.11	45.00
8	F	54.89	40.00
	R	54.78	45.00
9	F	54.92	40.00
	R	55.27	40.00

Table 2. Melting temperature and %GC

The secondary structure characteristics analyzed in this study were GC clamp, Cross Dimer, Hairpin, Self Dimer, and run (Table 3).

Primer pair		GC clamp	Cross Dimer (ΔG)	Hairpin (ΔG)	Self Dimer (ΔG)	Run
1	F	1	-6.47	-	-	3
	R	1		-	-6.36	-
2	F	2	-6.59	-	-	3
	R	2		-	-7.06	3
3	F	2	-6.94	-	-5.36	-
	R	2		-	-8.05	3
4	F	1	-6.46	-	-	3
	R	3		-	-	5
5	F	0	-12.86	-	-4.42	5
	R	1		-0.43	-4.53	3
6	F	1	-5.47	-	-	3
	R	2		-	-4.52	3
7	F	1	-5.88	-	-4.17	4
	R	1		-0.82	-3.94	-
8	F	1	-4.29	-	-	5
	R	0		-	-5.85	-
9	F	1	-6.82	-	-	3
	R	0		-	-5.85	3

Table 3. Secondary structure

IV. Discussion

The primer length generated from this calculation was 20 nucleotides. According to [6] designing primers for specific genes requires primer lengths ranging from 18 to 24 bp. Primer length plays important role in determining the annealing time in the PCR reaction. Primers that are too short will reduce the specificity of the primers, on the other hand, primers that are too long will cause the PCR reaction to be ineffective. The primer lengths for both forward and reverse of the nine candidates are the same (20 nucleotides).

This primer length corresponds to the criteria for a good primer. The primers obtained are unique sequences of nucleotide bases and should be of a short size to minimize costs. Good primer design is essential for successful reactions. The length of the amplicon varied from 151-299 bp. The length of the product will be shown on the PCR result tape. This PCR product is a DNA fragment with a certain length that can be detected by the most common technique, namely electrophoresis on agarose gel with ethidium bromide.

The melting temperature (Tm) of the design results was in the range of 54.78°C-55.14°C. Tm is the temperature at which half the DNA double strands are separate. The Tm value will affect the denaturation temperature of the DNA double helix strand and the primer annealing temperature. Primers with Tm too high above 70°C will easily mispriming at low temperatures. In addition, the formation of a bond that is too strong between the DNA template and the primer will result in low PCR products[7]. While primers with low Tm will not be able to work at high temperatures. All primary Tm design results in this study have met the reference in the program. In both primers (forward and reverse) there were differences in Tm values ranging from 1°C. The difference in Tm values between the two primers should not exceed 5°C. This will ensure that a suitable and specific annealing temperature is obtained in the PCR process.

The GC content of the primer design results was in the range of 40% -45% (Table 2). A good % GC value is in the range of 40-60%. The GC content will affect the PCR annealing temperature. To break the three hydrogen bonds Guanine and cytosine require a lot of energy and high temperatures[8].

GC Clamp is the number of bases G and C contained in the last 5 bases (3'). A good GC clamp is about 3 bases G or C and not more than 5 bases G or C. The presence of a G or C at the 3' end of the primer greatly assists in the stability of the bond between the primer and the template DNA required for polymerase initiation. The presence of a G or C base in the last five bases of the 3' end of the primer (GC Clamp) helps promote specific binding at the 3' end due to the stronger bonding of the G and C bases. The number of GC clamps in primer pairs 1 to 9 was in good criteria.

The PCR reaction should not contain secondary structures in the form of hairpins or dimer[9]. The stability of the secondary structure is determined by its free energy (ΔG) and its melting temperature. This causes the primer to be unable to adhere to the DNA template. The formation of a hairpin structure in the primer should be avoided, but it is very difficult to obtain a primer without a hairpin structure. The hairpin at 3' end with G(energy required to break hairpin structure) = -2 kcal/mol and the internal hairpin with G = -3 kcal/mol is still tolerable. Both primers should not have a T nucleotide base at the 3' end because it can cause a mismatch. Multiple mismatches or mismatches at the 3'-primary end can also cause hairpins.

Primers that bind to other primers of the same type are called self-dimers. Self-dimer at 3' end with G = -5 kcal/mol and self-dimer at the internal part with G= -6 kcal/mol is still tolerable. Primers that bind to their partner primers (reverse and forward) are called Cross-Dimers. The cross-dimer at the 3' end with G= -5 kcal/mol and the self-dimer at the internal part with G= -6 kcal/mol were tolerated.

Primers with long runs of a single base should generally be avoided as they can misprime. The maximum number of runs accepted is 4 bp. There are other factors that must be considered in primary specificity such as repeats which are sequences of dinucleotide repeats and runs which are sequences of nucleotide repeats. Primers are not allowed to contain three or more repeats and runs[10]. This is because the presence of repeats and runs will increase the possibility of false priming. The presence of false priming or primer attachment error outside the annealing temperature will result in incorrect product formation at a certain temperature so that the desired results are not appropriate. The primer pair chosen was number 6 because no hairpin structure in it and has a tolerable cross dimer and self dimer value and has less than 4.

V. Conclusion

A primer candidate has been selected from no 6. A primer has been designed to amplify the cellulase gene in *Bacillus cereus* with the forward primer ATGAACCACATCCAGAAACA and reverse primer TCTTTGTCCACTTCACGAAT. With the length of each primer (forward and reverse) was 20 nucleotides. The Tm in this primer were 54.84 ^{o}C and 54.92 ^{o}C, respectively. GC percent for forward and Reverse were 40%. This selected primer has 1 GC clamp on the forward and 2 GC clamp on the reverse.

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