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Primer Design of the Amylase Gene from *Bacillus cereus*

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Abstract:
Background*: Amylase is an enzyme that breaks down starch, turning it into sugar. There are two main types, namely alpha and beta. Amylase (α -amylase) is an enzyme that catalyzes the hydrolysis of alpha-1,4-glycosidic amylose starch to produce glucose. Among amylase, α-amylase has the greatest demand because of its various industrial applications such as the food, textile, paper, and detergent industries. Detection of the amylase gene on the PCR instrument requires a primer. The aims of research was to obtain the best pair of primers from the amylase gene designed in silico using the application of NCBI.*
Materials and Methods*: In this research, The search for the -amylase gene was conducted using the website http://www.ncbi.nlm.nih.gov, the National Center for Biotechnology Information (NCBI) database. Then the primary candidate screening was carried out using the same site. Then a forward and reverse primary analysis was performed*
Results*:* Of all these primary candidates, primary no 3 is the best primer
Key Word*:* α -amylase*; Bacillus cereus; Design ; Pcr ;Primer.*

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I. Introduction

Amylase (α -amylase) is an enzyme that catalyzes the hydrolysis of alpha-1,4-glycosidic amylose starch to produce glucose. Among amylase, α-amylase has the greatest demand because of its various industrial applications such as the food, textile, paper, and detergent industries[1]. α-amylase can be produced from plant or microbial sources [2 3 4] . Amylase from bacteria is widely applied in the industry because of its easy production. One of the bacteria that can produce amylase is *Bacillus cereus*[5]. Bacillus cereus is a Gram-positive, rod-shaped bacterium that can form spores. The most common sources found are liquid food products, powdered milk, mixed food products[6]. Detection of the amylase gene on the PCR instrument requires a primer in the form of a short-chain DNA sequence as a specific target DNA identifier. The primary design was carried out by insilico experiments It is necessary to design specific primers with insilico experiments so that the primers to be used are more specific in detecting the amylase gene in Bacillus cereus bacteria. This will make the amplification process by PCR more efficient and reduce the cost of using primers.. This research is a continuation of previous research [7] that aims to obtain the best pair of primers from the amylase gene designed in silico using the application of NCBI. This primer was designed to amplify the Alpha-amylase gene fragment in *Bacillus cereus* . Further experiments in the laboratory need to be carried out to determine the optimal conditions of this primer pair.

II. Material And Methods

α-amylase gene

The search for nucleotide sequences of the α-amylase gene from *Bacillus cereus* was carried out using data on the website http://www.ncbi.nlm.nih.gov, the National Center for Biotechnology Information (NCBI) database.

Screening of Primer Candidate

The nucleotide sequences that have been obtained at NCBI are then designed for primers using Primer-Blast, Pick Primer.

Primary Analysis Forward and Reverse

Primer candidate testing was carried out using data on the http://www.ncbi.nlm.nih.gov site to determine the sequence, length of each primer, start and stop position of the primer on the nucleotide sequence, melting temperature, percent GC, and length of the product. NetPrimer application on the considering the possible occurrence of hairpin structures, cross dimers..

III. Result

The search for nucleotide sequences of the alpha-amylase gene from *Bacillus cereus* was carried out using the data on the http://www.ncbi.nlm.nih.gov website using the keywords amylase and *Bacillus cereus* in the nucleotide box. Sequence data were obtained with the gene bank code EU735542.1, namely *Bacillus cereus* bacteria that can produce amylase as shown in Figure 1 below.

```
ORIGIN
        1 atgcttaaag aagccattta tcataggcca aaagataatt acgcatacgc ttacgatgaa
       61 aaaacgattc acatccgaat acgtacgaaa agagacgatg ttcaaagcac cactcttatt
      121 tatggtgatc cttatgaatg gaaagacggg aagtggattt cttcaagtac accgatgaaa
      181 aaaactggtt ctactgcatt gtttgattat tggttcattt ccatcgagcc gaaatttaag
      241 cgcttacgtt atggatttga attaaaaaac gatacagaca ctcttattta tgcagagcga
      301 ggatttttt ctaccactcc aaatgatgac gttggtaact ttttctgttt tccatttatt
      361 catgcaaatg atgtattcaa agcaccttct tggattaaag atactgtttg gtatcaaatt
      421 ttcccagaac ggttcgctaa tggagatcat acgctgaatc cagaaaacac ccttccttgg
      481 ggcagcgctg agccaactcc aactaatttt ttcggtggag attttgctgg tattattcaa
      541 aaccttgatt accttgttaa gcttggaatt tcaggaatat atttcacacc tattttcaaa
      601 gcacattcaa accataaata tgacacaatt gactacatgg aaatcgatcc acaatttggt
      661 acaaaagaaa cattcaaaga actcgttcag gcatgtcata cacacggtat aaaagtaatg
      721 ctcgatgctg tattcaatca cagtggatac tttttcgata aatttcaaga cgtactacaa
      781 aatggtgagc agtcagcata taaagaatgg ttccatattc atgagttccc aattagaact
      841 gagccactcc cgaattatga tactttcgcg tttacaccgt atatgccgaa attaaacaca
      901 gcgcacccgg atgtaaaaga atacttactt gaagtagggc gttattgggt gagagaattc
      961 aatatagacg gttggcgcct tgatgtagca aatgaagtcg accataactt ttggagagaa
     1021 tttcgaagtg agataaaagc attaaattct gaagtatata ttttaggaga aatttggcac
     1081 gatgcacttc catggctaca aggagatcaa ttcgatgctg tcatgagcta tcctgttaca
     1141 aacgccctac tgtcttactt tgctaacgat tccattaagg caaatgaatt tatgaaacaa
     1201 attacagaat ctctacattc ctactctatg aatgtaaatg aagcagcatt tcatttatta
     1261 gatagccatg atacaccaag aattttgaca acatgtaacg gagataaaaa taagttaaaa
     1321 ttactttatg tattccatct ttctttcatc ggctctcctt gtatttatta tggagacgaa
     1381 attggtatgg acggcggtat ggacccagat tgtcgcaaat gtatggtttg ggatacggaa
     1441 gaacaagatc atacattatt tacacatgta caaacattaa tttcattacg aaagcaatat
     1501 aaagcatttg gaggacatgg tactttccaa ttcattgaag caaatgatga atataattat
     1561 atttcttata cgaaaacata tgaagatgaa acgatctttt tcgttttaaa tccgactaat
     1621 agtgatatta cagcttcact ccctctccat gttactggaa agaaaatcat taatatttat
     1681 tacaaacgaa gaattttcag cggaagcaag tgtattacaa gttacacttc ctccaatgga
     1741 ttctccatat taaaatggta a
```

Figure 1. Nucleotides of *Bacillus cereus*

The nucleotide sequences were obtained from the NCBI. The primary design was carried out using PrimerBlast. The bacterium *Bacillus cereus* (gene bank code EU735542.1) was used as the template. 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 to a minimum of 50°C and a maximum of 60°C with an optimum temperature of 55°C as shown in Figure 2 below.

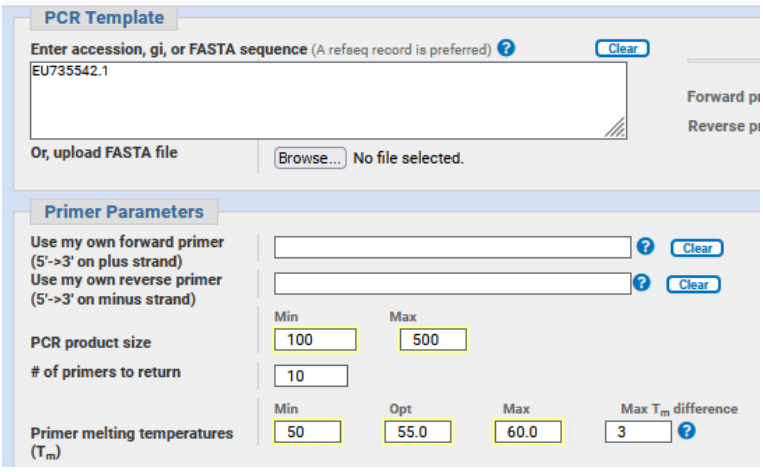
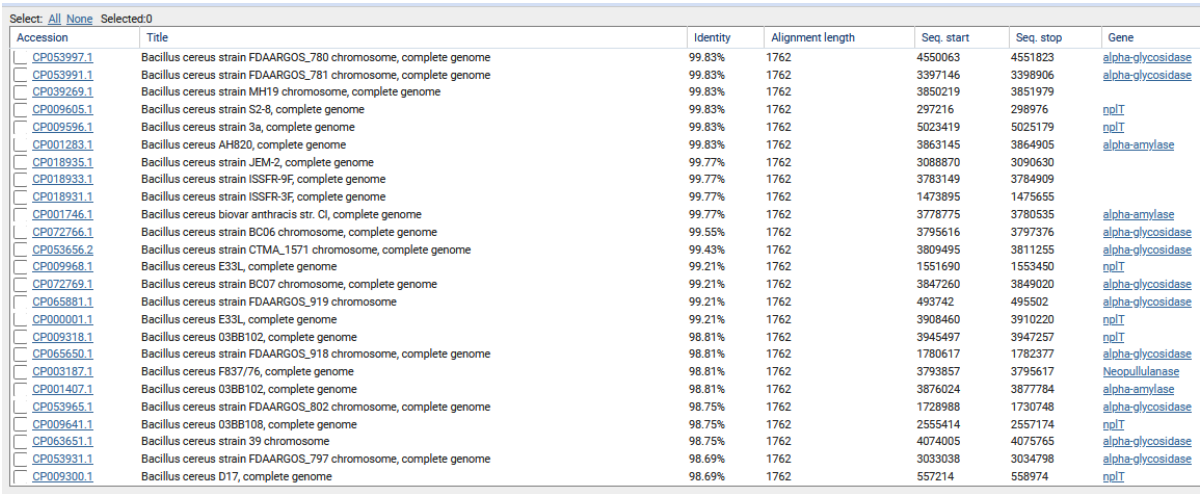


Figure 2. Primer template

Similar sequences appear after running to obtain primers from the template. From these sequences, it was traced to *Bacillus cereus* which had the amylase gene as the target gene (Figure 3).



Select: All None Selected:0

Accession	Title	Identity	Alignment length	Seq. start	Seq. stop	Gene
CP053997.1	Bacillus cereus strain FDAARGOS_780 chromosome, complete genome	99.83%	1762	4550063	4551823	alpha-glycosidase
CP053991.1	Bacillus cereus strain FDAARGOS_781 chromosome, complete genome	99.83%	1762	3397146	3398906	alpha-glycosidase
CP039269.1	Bacillus cereus strain MH19 chromosome, complete genome	99.83%	1762	3850219	3851979	
CP009605.1	Bacillus cereus strain S2-8, complete genome	99.83%	1762	297216	298976	nplT
CP009596.1	Bacillus cereus strain 3a, complete genome	99.83%	1762	5023419	5025179	nplT
CP001283.1	Bacillus cereus AH820, complete genome	99.83%	1762	3863145	3864905	alpha-amylase
CP018935.1	Bacillus cereus strain JEM-2, complete genome	99.77%	1762	3088870	3090630	
CP018933.1	Bacillus cereus strain ISSFR-9F, complete genome	99.77%	1762	3783149	3784909	
CP018931.1	Bacillus cereus strain ISSFR-3F, complete genome	99.77%	1762	1473895	1475655	
CP001746.1	Bacillus cereus biovar anthracis str. CI, complete genome	99.77%	1762	3778775	3780535	alpha-amylase
CP072766.1	Bacillus cereus strain BC06 chromosome, complete genome	99.55%	1762	3795616	3797376	alpha-glycosidase
CP053656.2	Bacillus cereus strain CTMA_1571 chromosome, complete genome	99.43%	1762	3809495	3811255	alpha-glycosidase
CP009968.1	Bacillus cereus E33L, complete genome	99.21%	1762	1551690	1553450	nplT
CP072769.1	Bacillus cereus strain BC07 chromosome, complete genome	99.21%	1762	3847260	3849020	alpha-glycosidase
CP065881.1	Bacillus cereus strain FDAARGOS_919 chromosome	99.21%	1762	493742	495502	alpha-glycosidase
CP000001.1	Bacillus cereus E33L, complete genome	99.21%	1762	3908460	3910220	nplT
CP009318.1	Bacillus cereus 03BB102, complete genome	98.81%	1762	3945497	3947257	nplT
CP065650.1	Bacillus cereus strain FDAARGOS_918 chromosome, complete genome	98.81%	1762	1780617	1782377	alpha-glycosidase
CP003187.1	Bacillus cereus F837/76, complete genome	98.81%	1762	3793857	3795617	Neopullulanase
CP001407.1	Bacillus cereus 03BB102, complete genome	98.81%	1762	3876024	3877784	alpha-amylase
CP053965.1	Bacillus cereus strain FDAARGOS_802 chromosome, complete genome	98.75%	1762	1728988	1730748	alpha-glycosidase
CP009641.1	Bacillus cereus 03BB108, complete genome	98.75%	1762	2555414	2557174	nplT
CP063651.1	Bacillus cereus strain 39 chromosome	98.75%	1762	4074005	4075765	alpha-glycosidase
CP053931.1	Bacillus cereus strain FDAARGOS_797 chromosome, complete genome	98.69%	1762	3033038	3034798	alpha-glycosidase
CP009300.1	Bacillus cereus D17, complete genome	98.69%	1762	557214	558974	nplT

Figure 3. Sequences with close similarities

From the data obtained, there are 10 pairs (forward-reverse) of primary candidates after the selection of sequences with the target gene (figure 4).

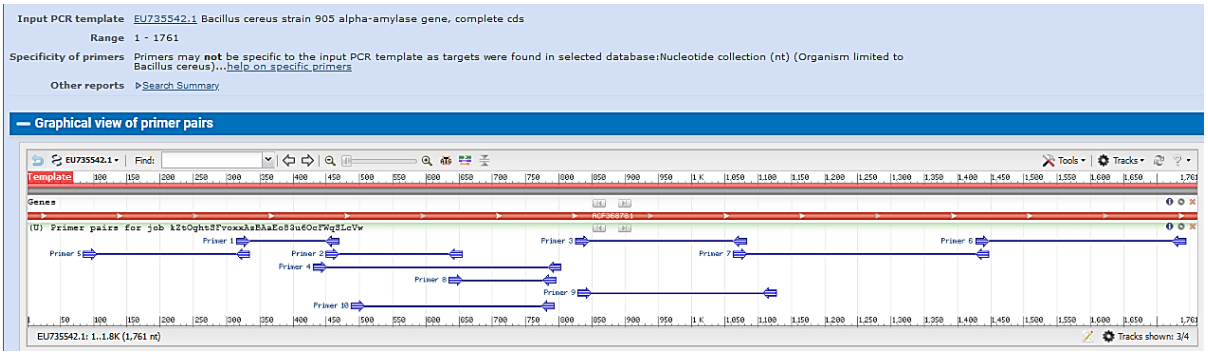


Figure 4. Primer candidate

The primer candidate determination was carried out using data on the http://www.ncbi.nlm.nih.gov site to determine the sequence of each primer, product length (Table 1), length of each primer, start position and position primary stop on nucleotide sequence, melting temperature, percent GC (Table 2)

Table 1. Length of Product

Primer Pair	Sequence (5'->3')		Product length
1	F	CACTCCAAATGATGACGTTG	155
	R	TGTTTTCTGGATTCAGCGTA	
2	F	TACGCTGAATCCAGAAAACA	205
	R	TTGTGGATCGATTTCCATGT	
3	F	TTCCCAATTAGAACTGAGCC	257
	R	TCGTGCCAAATTTCTCCTAA	
4	F	GGTTCGCTAATGGAGATCAT	373
	R	TTATATGCTGACTGCTCACC	
5	F	ACGAAAAGAGACGATGTTCA	250
	R	CAACGTCATCATTTGGAGTG	
6	F	TTGGGATACGGAAGAACAAG	316
	R	GAATCCATTGGAGGAAGTGT	
7	F	TTAGGAGAAATTTGGCACGA	385
	R	CTTGTTCTTCCGTATCCCAA	
8	F	ACATGGAAATCGATCCACAA	161
	R	TGACTGCTCACCATTTTGTA	
9	F	CCAATTAGAACTGAGCCACT	299
	R	CTCATGACAGCATCGAATTG	
10	F	CTGAGCCAACTCCAACTAAT	306

	R	ACTGCTCACCATTTTGTAGT	

Table 2. Characteristics of Primer

Primer Pair	Sequence (5'->3')		Length	Start	Stop	Tm	GC%
1	F	CACTCCAAATGATGACGTTG	20	315	334	55.01	45.00
	R	TGTTTTCTGGATTCAGCGTA	20	469	450	55.01	40.00
2	F	TACGCTGAATCCAGAAAACA	20	450	469	55.01	40.00
	R	TTGTGGATCGATTTCCATGT	20	654	635	55.03	40.00
3	F	TTCCCAATTAGAACTGAGCC	20	826	845	55.03	45.00
	R	TCGTGCCAAATTTCTCCTAA	20	1082	1063	54.97	40.00
4	F	GGTTCGCTAATGGAGATCAT	20	431	450	55.01	45.00
	R	TTATATGCTGACTGCTCACC	20	803	784	54.94	45.00
5	F	ACGAAAAGAGACGATGTTCA	20	85	104	55.07	40.00
	R	CAACGTCATCATTTGGAGTG	20	334	315	55.01	45.00
6	F	TTGGGATACGGAAGAACAAG	20	1428	1447	55.08	45.00
	R	GAATCCATTGGAGGAAGTGT	20	1743	1724	55.02	45.00
7	F	TTAGGAGAAATTTGGCACGA	20	1063	1082	54.97	40.00
	R	CTTGTTCTTCCGTATCCCAA	20	1447	1428	55.08	45.00
8	F	ACATGGAAATCGATCCACAA	20	635	654	55.03	40.00
	R	TGACTGCTCACCATTTTGTA	20	795	776	54.89	40.00
9	F	CCAATTAGAACTGAGCCACT	20	829	848	55.05	45.00
	R	CTCATGACAGCATCGAATTG	20	1127	1108	54.88	45.00
10	F	CTGAGCCAACTCCAACTAAT	20	488	507	55.05	45.00
	R	ACTGCTCACCATTTTGTAGT	20	793	774	54.88	40.00

Observations were made on the possibility of hairpin structures, cross dimers (Table 2) using the NetPrimer application.

Table 3. Secondary Structure

Primer Pair		Hairpin (kcal/mol)	Cross Dimer (kcal/mol)
1	F	-0.7	-6.6
	R	*	
2	F	*	-8.07
	R	-3.3	
3	**F**	*****	**-5.5**
	R	*****	
4	F	-0.03	-5.41
	R	-0.03	
5	F	-0.02	-6.53
	R	*	
6	F	*	-6.12
	R	-0.34	
7	**F**	*****	**-7.97**
	R	*****	
8	F	-3.3	-6.5
	R	*	
9	**F**	*****	**-8.32**
	R	*****	
10	**F**	*****	**-8.27**
	R	*****	

IV. Discussion

The primer design required for PCR is a pair of primers known as a forward primer and a reverse primer. The primer obtained is a unique series of nucleotide bases and is attempted to have a short size to minimize costs. A good primer is a primer that meets the criteria for primer parameters. These parameters include: primer length, melting temperature (Tm), percentage of the amount of G and C (%GC), and hairpins. The results of the analysis obtained 10 pairs of primer candidates with different product lengths (Table 1). Each primer consists of 20 nucleotide base lengths (Table 2). The results of the primer design meet the criteria for

good primer parameters. The specificity, temperature, and annealing time depend on the primary length. Primers with 18-24 nucleotide lengths were a good measure of sequence-specific if the PCR reaction annealing temperature was set within 50°C of the primer-template temperature[8]. PCR primers that are too short will tend to experience mispriming and primers that are too long have the potential for hybridization to occur so that it will inhibit the DNA polymerization process[9]. Choosing the right primer for PCR is an important factor. The strength of the primer-template bond in the forward and reverse greatly affects the formation of the PCR reaction. Primers must not have a match with other targets so that our target amplification process becomes more specific. Compatibility in terms of a certain orientation as well as a certain distance that allows unwanted amplification. The primer sequence should not have a region that can bind to the internal primer or to its partner primer. Primers should use complementary base pairs and not have many binding sites in the target genome.

The melting temperature (Tm) on the primer is in the range of 50°C-60°C. Tm is the temperature at which half the DNA double strands 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[10]. Meanwhile, primers with low Tm tend to stick to other places and produce non-specific products. From the 10 candidate primers obtained, it can be seen that all of the primers, both forward and reverse, were in the Tm range. The difference between Tm forward and Tm reverse does not exceed 5^oC. It can be concluded that all the candidates meet the criteria for a good primary Tm.

The percentage of good GC ranges is from 40-60%. GC is the content of the number of bases G (guanine) and C (cytosine) which can affect the Tm of a primer, besides that the percentage of GC also affects the bonding between strands in DNA. High GC content will complicate the separation of double-stranded chains in primers and templates[11]. From the design results obtained GC values that meet the criteria.

The stability of the primer determines its false priming efficiency with a stable 5' end and an unstable 3' end. If the primer has a stable 3' end, it will bind to a complementary site other than the target with the 5' end dangling over the edge. This can then lead to a secondary band. Stability of the 5' termini allows for efficient bonding of the primer to the target site[12]. The formation of a loop/hairpin structure in the primer should be avoided, but it is very difficult to obtain a primer without a hairpin structure. Based on table 3, the primer candidate's numbers 3,7,9,10 didn't have a hairpin. A hairpin loop is formed when the primer folds back upon itself and is held in place by intramolecular bonding. Because hairpin loop formation is an intramolecular reaction, it can occur with as few as 3 consecutive homologous bases [12]. Primers that bind to their partner primers (reverse and forward) are called Cross-Dimers. The cross-dimer in primer no. 3 has the best value among no. 7,9, and 10 with the largest G so that it can still be tolerated[13]. Of all these primary candidates, primary no 3 is the best primer

V. Conclusion

A primer has been designed to amplify the Alpha amylase gene in Bacillus cereus with the forward primer TCCCAATTAGAACTGAGCC and reverse primer TCGTGCCAAATTTCTCCTAA. With the length of each primer (forward and reverse) was 20 nucleotides. The Tm in this primer were 55.03 and 54.97^oC, respectively. GC percent for forward 45% and Reverse 40%.

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