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Cloning and overproduction of chymosin from *Bacillus* spp in *Escherichia coli* origami

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ABSTRACT

Making cheese is simply a process of turning a liquid (milk) in-to a solid. Rennet has an important role in this process which is a complex set of enzymes where chymosin is the key component. The aim of this study was identifying the *Bacillus* genus with the ability of producing the enzyme. 50/75 isolates from soil of Gandom Beryan area of Kerman, Iran, determined as bacillus spp via classical and PCR methods. The presence and over production of the cltA gene (encoding chymosin) were investigated by performing cloning in *Escherichia coli (E. coli) origami* and functional cloning confirmed by using real-time reverse transcription – polymerase reaction (Real-time PCR) and clonal selection. The results showed functional cloning of the gene in *Escherichia coli* origami. Among 50 isolates of *Bacillus* spp, 4 isolates (8%) showed the capacity to produce the gene. Phylogenetic characterization analysis of 16S rRNA gene of the *Bacillu*s spp determined the strains as *Bacillus cereus*. According to the results of this study, expression of the gene via Real-time PCR showed their ability to produce clt A gene and rennet. Although to further confirmation, a larger samples needs to be performed and additional tests are required. Due to animal rennet deficiency, determining such accessible and cheaper sources in producing rennet is important.

1. Introduction

Rennet is a complex set of enzymes mostly chymosin or rennin but it has some other enzymes like pepsin and lipase. Use of rennet during chees making usually considered as curd. The origin of the discovery of rennet was the abomasa mucosa of new born calf and lamb vells (Camin et al., 2019). It can be derived from three sources (El-sohamiy and El- saadani, 2010) like animal rennet, aspartic acid proteinases of microbial and plant origins (Soodam et al., 2015). Animal rennet is traditionally used as a milk–coagulant in food industries to produce high quality cheese (Poza et al., 2004) though its price has been growing because of the reduction in the supply of calf rennet (Mostafa et al., 2013). Use of biologic sources is one of the best and affordable methods (Alecrim et al., 2015), but producing the enzyme from microorganisms cannot meet the business needs (Handelsman et al., 2015). Hence using cloning with the aim of more production and achieving a better enzyme quality is favorable. Genetic chymosin is produced through fermentation by cloning in a host microorganisms in which the *clt* gene for the enzyme is expressed. This made recombinant

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DNA technique dealing with microbes more popular (Martinen et al., 2012). Chymosin can be produced in large-scale fermenters at low cost using microorganisms. The most common microorganisms used to produce recombinant chymosin are fungi such as Aspergillus oryzae, Rhizomucor pusillus (Soodam et al., 2015). Camin et al (2019) worked on fermentation-produced chymosin. They explained that the function of this kind of chymosin is similar to animal one but is much cheaper. There are some studies about the role of bacteria which have also been studied for their capacity for producing chymosin (Sadeghi et al., 2016). Interestingly, microbial enzymes which have commercial capability, were mostly found in *Bacillus* spp (Avsar et al., 2017). Mohammadi et al., 2016 reported that *Bacillus cereus* and *Bacillus subtilis* have suitable ability in pectinase production. *Bacillus cereus* is one of the accessible soil bacteria that has never been studied for producing rennet so far. Because of microbial diversity of soils in different geographical areas, the aim of this study was to find these cheaper sources with the ability of producing cltA gene. In present study, we reported the successful cloning of *clt* gene from local *Bacilli*. The main focus was isolating and determining the *Bacilli* which carried *clt* gene from Gandom Beryan area and cloning the isolates in *E. coli* origami using PCR and recombinant DNA technique.

2. Materials and Methods

2.1. Bacterial strains

75 samples of soil bacteria were collected from Gandom Beryan area of Kerman, Iran and cultured in nutrient agar with pure plate method, incubated at 37°C for 48 hours. Then by using gram stain, malachite green and biochemical tests (including methyl red, voges proskauer, simmons citrate agar, oxidase catalase, urease, triple sugar iron agar and nitrate reduction), bacillus genus were identified (using Bergey `s Manual of Determinative Bacteriology) (Lucas et al., 2006).

Molecular identification of Bacillus genus

Bacillus spp was identified by phenotyping and sequencing of 16S rRNA. The primers used in this step were as follows: 16s forward: 5'-AGA GTT TGA TCC TGG CTC AG-3' size = 1500bp

16s reverse: 5'-AAG GAG GTG ATC CAG CCG CA-3' size= 1500bp

2.2. Extraction of DNA

DNA extraction was done according to the protocol of DNA Extraction Kit (Lot: No. 802136, SINA COLON. Co, Iran) as mentioned in the manufacturer's instructions. The extracted DNA was evaluated by Promega kit (Lot: No. 000026093). The Quality of the DNA was measured by running DNA on gel agarose, placed on ethidium bromide for 15 min, and visualized on UV-trans illuminator.

2.3. PCR amplification

By using sequences of various *cltA* gene from NCBI, degenerative primers for *cltA* gene were designed and synthesized by Takapouzist. co. Iran. The primers used in this step are as follows: *clt* A forward: 5-′ATG CCC ATG TTC AAG AAG ATG-3′size= 798 bp.

clt A reverse: 5′-TCA GTA CAG GTA CTG GAG CGC-3′ size=798 bp

Amplification was performed in a thermal cycler, using a total volume of 20 µl, consisting of Master mix (10 µl) , primers (1 µl of each) , DNA template (3 µl) and 5 µl of distilled water.

PCR amplification was performed with initial denaturation at 95°C for 5 min and 35 cycle including denaturation 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. PCR products were run on agarose gel 1%, visualized on UV-trans illuminator, photographed via a gel documentation system with a 100 bp ladder used as molecular marker.

2.4. Plasmid, Cloning of the cltA gene

The *Escherichia coli* origami used in this study was received from university of Tehran and cultured in LB medium at 37°C 16-17 hours. It was centrifuged for 10 min at 10000 Rpm,100 µl of buffer poured in to precipitate and 100 µl of this complex was added to ligation solution and placed in ice for 30 min. After that it was subjected to temperature of 42°C for 90sec and reserved in 1.5 µl tubes. Cloning was performed according to the instruction of PCR TA cloning kit (Lot, No, CL5841, SINA COLON.Co, Iran). *PTG19-T* A cloning was used as the expression

vector in this study. The ligation was performed with 10 µl of mixture including the vector (2 µl) ,fresh and purified PCR product (1.5 µl), 10x buffer ligase (1 µl) , T4 DNA ligase (0.6 µl) and sterile Deionized water (4.9 µl). This mixture was used to transform the gene into the vector.

The PCR amplification of reaction mixtures was done as follows: initial denaturation step at 95°C for5 min, denaturation at 94°C for 30 sec, annealing at 55°C for 45sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. Then 100 µl of the recombinant plasmid was cultured on LB agar containing 50 mg/ml of ampicillin, 160 µl IPTG and 160 µl Xgal.

2.5. Confirmation of successful cloning

The correct orientation of inserted fragment was confirmed, Sequence initialization was accomplished with the universal primers M13 forward and revers, and oligonucleotides were later designed as the sequence proceeded. The following primers were used at this stage (Elsayed et al., 2010). 5'-CCA GGG TTT TCC CAG TCA CGA-3'= M13 forward. 5'-CAC ACT TTA ACA ATA GGC GAG-3'= M13 Reverse. The selected colonies were analyzed by Real-time PCR.

2.6. Real-time PCR

The suspension of recombinant colony in the late logarithmic phase (OD600=0.4, 0.6) was used in this step. RNA extraction was done based on the instruction of the RNA extraction kit (Lot: No, 9560325) while the quantitative evaluation of RNA was done by Promega kit (Lot, No:0000274021) and Floro meter E6150. Then the extracted RNA was incubated at 65°C for 3 min and cDNA was synthesized with the concentration of 25 lµ/unit *Reverse AMV* enzyme (received from Roche Company). Real-Time PCR reaction was performed by a total 20 µl concentration including Prime Q master mix with cyber green (10 µl) , primers (1 µl of each primers), Rox dye (1 µl) cDNA (2 µl) and water. A housekeeping 16srRNA was used as endogenous control. Amplification was performed by the instruction of the Genet bio kit (No: Q9210, south Korea. At the last step, the product was sent to Takapouzist.co for sequencing. Gene expression analysis was performed by relative measurement of mRNA expression compared to standard strains.

The sequences were analyzed by Bio Edit software. Then the two sequenced strains(forward/reverse) were arranged from 5´to 3´ by means of DNA Baser software. The sequences were compared in NCBI and Gene Bank (https://www.ncbi.nlm.nih.gov/genbank/) and aligned with the W ClusTA1 software. Phylogenetic tree analysis was performed by *MEGA7*.

3. Results

The results of gram stain, malachite green and biochemical tests (including methyl red, voges proskauer, simmons citrate agar, oxidase catalase, urease, triple sugar iron agar and nitrate reduction are shown in table 1. Among 75 samples, fifty of them (50/75) were identified as *Bacillus* spp that then were determined as *Bacillus cereus* based on phylogenetic characterization analysis of its 16srRNA.

3.1. Presence, cloning and expression of clt gene

The presence of clt gene encoding chymosin were amplified in *Bacillus cereus* via PCR method on the 1.5% agarose gel, the results was positive for 4 (4/50) isolates on the 798 bp band (Fig.1).

Cloning into PTG19-T vector, the gene was successfully expressed in *Escherichia coli origami* upon induction with IPTG. Successful cloning was confirmed using PCR with the M13 primers. The quantity of extracted RNA manifested the increasing size of the product from 798 bp to 991bp (Fig.2) that showed the successful entrances of recombinant bacteria in to *Escherichia coli origami* and the results of clonal selection was shown in Fig.3a confirmed the cloning too.

Real-time PCR determined the overexpression of the *cltA* gene in *Escherichia coli* origami. The expression of gene was confirmed by formation of 23s band (Fig.3b). The Results of Phylogenetic tree based on 16s RNA sequences was shown in Fig.4. As indicated in the phylogenetic tree the *Bacillus cereus* and *Bacillus subtilis* have the same phylogenic cluster.

Table 1. The results of biochemical tests of *Bacillus* spp

S.No.	Test	Response of the organism
1.	Gram staining	+
2.	Methyl Red	+
3.	Voges proskauer	+
4.	Simmons citrate agar	+
5.	Oxidase	-
6.	Catalase	+
7.	Indole	-
8.	Urease	-
9.	Triple Sugar iron Agar	+
10.	Nitrate reduction	-

(+ = Positive reaction, - = Negative reaction)

From these tests 50 of isolated bacteria determined as *Bacillus spp*

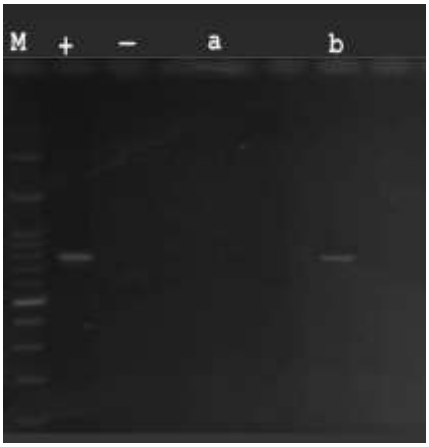

Fig 1. Results obtained for *cltA* gene in *bacillus sereus* on the gel agarose 1.5%. DNA molecular weight markers. C- distiied water. Lane a. negative results of presence of clt gene for 46 isoltes. b. The positive results of 798 bp fragment of clt gene were observed in 4/50 (8%) bacillus spp (isolated number 3,16,31,37), Lane

Fig 2. The results of functional cloning by PCR with M13 primer. a. Positive results of PCR of the extracted DNA of new recombinant colonies, The product size was increased after cloning, Lane 1. Befor cloning (798 bp). Lane 2. After cloning (991 bp).

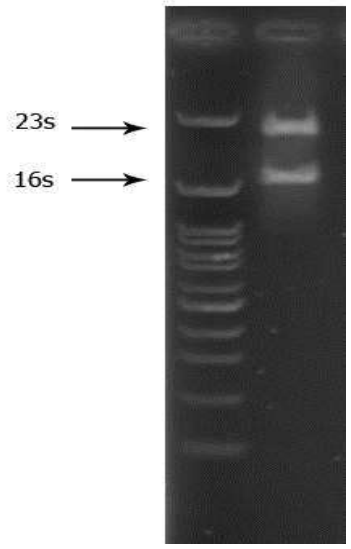

Fig 3 a,b. confirmation of functional cloning and overproduction of *clt* gene.

a. Clonal selection (white and blue) results. The formation of white colonies showed successful cloning.

b. RNA qualitative evaluation showed here, the increasing size of Real-Time PCR from 16s to 23s band, confirmed the cloning.

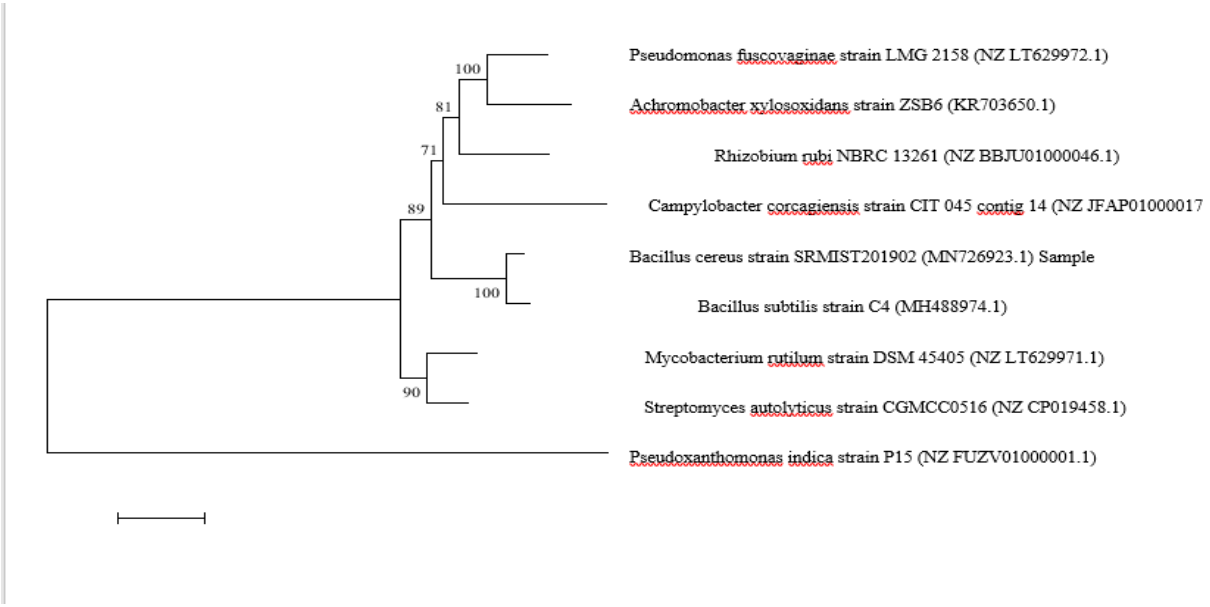

Fig 4. Results of Phylogenetic tree based on 16s RNA sequences. The data reveal similarity between *Bacillus cereus* and *Bacillus subtilis*.

4. Discussion

Cheese is a favorable consumed fermented dairy product with an increasing consumer demand. It is valued for its portability and high composition of protein, fat and calcium (Lucas et al., 2006; Dutt et al., 2009). Due to advent of genetic engineering and use of recombinant DNA, important genes could be cloned in microorganisms such as *E.coli*. In this study the clt gene was identified, sequenced and cloned into PTG19 vector, transformed in to *Escherichia coli* origami to produce overproduction and safer chymosin. *Bacillus* spp have variety roles in biotechnology and industry. Because of difficulty in characterizing new strains, molecular methods are used for quick species identification (Kumar et al., 2014) so in

this study the *Bacillus* isolates were determined as *Bacillus cereus* via Phylogenetic characterization analysis of 16S rRNA.

To select *bacillus* spp with the ability to produce clt gene encoding chymosin, 75 soil bacteria from Ganom Beryan area in kerman, Iran, were collected. 50/75 were determined as *bacillus* spp which four of them carried the gene. The results of this study showed soil bacilli′s capacity for producing of *clt* gene, which confirmed the previous researches about high capacity of microorganisms in producing the enzyme (Fitriani and saksono, 2010).

E. coli is a best understood microorganism. It is a key tool in molecular genetics (liu et al., 2020) so cloning into *E. coli* vector plasmids is a standard procedure in most molecular biotechnology laboratories and former studies used it as a best host. Because of the reason mentioned above and low frequency of *Bacillus cereus* transform as a first host, in this study we use *Escherichia coli* origami for functional cloning. It is noticeable that in present study, we used TA cloning, a faster method than classical cloning that used in previous studies with no use of restricted enzymes.

qRT-PCR is known as the effective method to analyze gene expression due to its efficiency to detect and quantify the target genes. Previous studies have shown that isolated *Bacillus* had the capacity for producing large amounts of protease (Berridge, 1942). The results of this study showed the ability of 8% of the *Bacillus cereus* to produce the enzyme that manifested the capacity of the local bacillus cereus in producing cltA gene was also revealed in this study. Chen *et al* (2010) reported in their study that the *Bacillus subtilis* spp have the ability to produce rennet, and manifested the highest proteolytic activity of the species (Chen et al., 2010). The results of another study performed by Ding *et al* (2011) showed the *Bacillus subtilis* ability in production of milk – clotting enzyme (Ding et al., 2011). In present study *Bacillus cereus* isolates were investigated for such ability that proved *Bacillus cereus* has the ability of producing the enzyme. The results of this study was in harmony with other studies about the importance of microbial rennet. More ever in present study we reported that *Bacillus subtilis* and *Bacillus cereus* were placed in the same cluster where alignment was recognized between them.

Conclusion

In sum, this study describes cloning and over production of *clt* enzyme from local *Bacillus cereus* in *E. coli origami*. Because of high diversity of soil microorganisms and their capacity for producing the enzyme, soil *Bacilli*, carrying *clt* gene were isolated by molecular method. For better cloning we used TA cloning method that conserves time and is affordable economically. By using PTG19-T as cloning and expression vector and performed the ligation, new recombinant plasmid were tested by Real- time PCR and sequencing of products.

Because of little studies about *Bacillus* spp, especially *Bacillus cereus* in producing microbial rennet and considering the high demands for rennet, the shortage of animal rennet, and the accessibility and easy culturing of *Bacillus cereus*, it is important to identify and isolate this genus which can provide rennet in large amount with low cost. Producing rennet by the *Bacillus cereus* used in this study that was done for the first time and successful cloning of it in *Escherichia coli* origami may encourage to do more research in order to find other genus with such ability. The results of this study showed the capacity of the *Bacillus cereus* isolated from Gandom Beryan area of Kerman, Iran, for producing the *cltA* gene, which was the main focus of this study but a larger samples needs to be performed and additional tests are required to confirmed these results.

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Conflict of interest:

There is no conflict of interest

Refereces

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