



RESEARCH PAPER

OPEN ACCESS

Cloning and expression of *Bacillus licheniformis amyS* gene in *E. coli* BL21 (DE3)

Ashraf Kariminik^{1*}, Kobra Saeidi¹, Sayed Mohammad Reza Khoshroo¹

¹Department of Biology, Faculty of Science, Kerman Branch, Islamic Azad University, Kerman, Iran

Key words: *Bacillus licheniformis*, pET-26b (+), recombinant protein, alpha-amylase.

<http://dx.doi.org/10.12692/ijb/4.11.316-323>

Article published on June 11, 2014

Abstract

Alpha amylase is an endoamylase enzyme that hydrolyzes amylose, amylopectin and glycogen by randomly cleaving internal alpha 1, 4 linkages. This enzyme is widely distributed in various bacteria, fungi, plants and animals. Microbial cells, because of their diversity in nature, are usually used in amylase production. The genus *Bacillus* is one of the most common sources of alpha- amylase production. The aim of this study was to identify the overexpression and activity of *Bacillus licheniformis* α -amylase. The sequence related to alpha-amylase production was determined by using polymerase chain reaction technique. Certain fragment was introduced to the expression vector pET-26b (+). After sequencing, recombinant vector was introduced to *E.coli* BL21 (DE3) and expressed. Initially, the enzyme was expressed as insoluble solid objects that had a molecular weight (based on SDS-PAGE) equal to the predicted values. The recombinant enzyme was purified by heat treatment at 80 °C for 20 minutes and was filtered by using dialysis bags. Alpha -amylase activity was measured by employing the Di-nitro Salicylic Acid technique.

* Corresponding Author: Ashraf Kariminik ✉

Introduction

Enzymes are widely used in food production and their applications are an important issue in various sciences such as food science, medicine, pharmacy and in industries related to these sciences (Pandey *et al.* 2000, Tucker and Woods 1995). The most important groups of enzymes with numerous applications are amylase group (Windish *et al.* 1965). These enzymes hydrolyze starch, catalyze glycogen (Universiteit 1993). The history of amylases initiated with the discovery of first starch-degrading enzyme in 1811 by Kirchoff (Sarbatly and England 2004). Amylases derived from microbial sources are divided into groups of bacterial and non-bacterial and heat resistant and ordinary groups as well (Maarel and Marc 2002). Bacterial α -amylases (α -1,4-glucan-4-glucanhydrolase) are secreted proteins that hydrolyze α -1,4 glycosidic linkages, when starch is the sole carbon source (Alariya *et al.* 2013). Most alpha-amylases are stable in a wide range of pH values in the presence of calcium ions (Gupta *et al.* 2002). The presence of calcium is necessary for the activity and stability of these enzymes and increases the resistance of alpha-amylases to heat (Kuriki and Imanaka 1999, Vinhinen and Mantsala 1989). Alpha-amylases are among the most important enzymes used in the starch industry including brewing, baking and jam making (Roy and Mukherjee 2013, Ozcan *et al.* 2001). Nowadays, alpha-amylases are utilized in industries such as food industry, detergents, textiles, and in paper industry to hydrolyze starch (Rana *et al.* 2013). Amylases constitute the largest share (i.e. 30 percent) of the world trade in enzymes (Reddy *et al.* 2003). The genus *Bacillus* produces a large diversity of extracellular enzymes, some of which are of industrial importance. *Bacillus licheniformis* is a Gram-positive rod-shaped bacterium (Dewaliya and Jasodani 2013a). It tends to form spores in soil which makes it desirable to be used for the industrial purposes such as the production of antibiotics (Smitha and Bhat 2013), Biosurfactants (Dewaliya and Jasodani 2013b) and enzymes such as alkaline protease (Sathyavathan and Krithika 2014, Sellami-Kamoun *et al.* 2008) and amylases (Rothstein *et al.* 1986). *Bacillus licheniformis* α -amylase (BLA) has widely used in

starch liquefaction processes because of its strangely high thermal resistance (Declerck *et al.* 2002, Lonsane and Ramesh 1990). In this study, the α -amylase gene (*amyS*) from *B. licheniformis* (GenBank Accession Number M13256) is expressed in *Escherichia coli* BL21, and the expression of this recombinant enzyme by *E. coli* secretory system and activity of the enzyme is discussed.

Materials and methods

Bacterial sample

B. licheniformis, the bacterial strain used in this work, was obtained from the technological and scientific research center in Tehran, Iran. Microorganism Database in this center is characterized as PTCC 5027.

DNA extraction

DNA extraction was performed by using CinnaGen gram positive DNA extraction kit (Cat.No.PR881614).

Primer design

The primer was designed by using the Oligo 5 ver. 2.00 program. The SignalP program (<http://www.cbs.dtu.dk/services/SignalP>) was first used to identify the signal peptide of the related protein and, after omitting it, the forward primer was designed. The terminal codon of the related gene was also taken into consideration in designing the reverse primer. Therefore, the recombinant protein lacked the His tag region.

The primers used had the 5' AGTGGCCATGGCAAATCTTAATGG 3' sequence as the forward primer and the 5' TCTGCTCGAGCTATCTTTGAACATAAAATTG 3' sequence as the reverse primer.

Polymerase chain reaction

The nutrient broth medium was used to culture the bacteria, and the DNA extraction kit manufactured by the CinnaGen Company was employed to extract the genomic DNA. The PCR technique, with the help of selective primers, was used for selective replication of fragments. The annealing temperature of 55 degrees

centigrade with the duration of one minute was selected for this reaction. The replicated fragment was purified by using the purification kit manufactured by the CinnaGen Company as specified by the company in the directions.

Gene cloning

Cleaving of the expression plasmid pET-26b (+) by the enzymes *Xoh1* and *Msc1* took place for 12 hours at 37 °C. At the end of the cleaving operation, the digested plasmid was purified. The PCR products and the plasmids were joined together with the help of the enzyme T4 DNA ligase and re-transformed into circular plasmids. The *E. coli* MC 1061 bacteria was used to prepare competent cells for transferring the cloned fragments. Twenty microliters of the products of the ligation reaction were added to 150 microliters of competent bacterial cells. Finally, the prepared cells were placed in the LB culture medium containing kanamycin for 12 hours at 37 °C to complete their growth and be ready for confirmation steps (Torry 1983). To identify bacteria containing the desired fragment, the direct PCR technique was used with the help of T7 promoter primer and T7 terminator primer. After selecting bacteria containing the desired fragment, the plasmids in MC1061 bacteria were extracted and transferred to *E. coli* BL 21 (DE3) bacteria.

Recombinant alpha-amylase expression

In order to express recombinant plasmid, the desired bacteria were first cultured in the liquid LB culture medium containing the antibiotic kanamycin (20 mg/mL) for 12 hours at 37 °C, with the culture medium rotated at 150 revolutions per minute (rpm). 10 milliliters of the cultured bacteria were then added to 100 milliliters of the liquid TB culture medium containing kanamycin and the culture medium was rotated at 180 revolutions per minute (at the same temperature). After the bacteria had grown for four to five hours, 50 microliters of 1 M IPTG (with the final concentration of 0.5mM) were added to the culture medium and the bacteria were cultured for four more

hours. The culture medium containing the bacteria was then centrifuged at 6000 revolutions per minute (4629 g) for 10 minutes at four degrees centigrade and the supernatant liquid was then discarded. Ten milliliters of the TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.8) were added to the remaining precipitate and the samples were mixed and kept at - 20 °C until the next steps were carried out. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to observe the abundant expression of the desired plasmids.

Preparation of the periplasmic extract

Lysozyme (final concentration 1mg/ml) and one tablet of complete protease inhibitor cocktail were added to the cell suspension, followed by adding 200ml of ice cold deionized water. The suspension was agitated at 60rpm on ice for 1h and the cells were centrifuged at 16000 g for 20 minutes. Both the pellet as the cytoplasmic and membrane fractions and the supernatant, which constituted the periplasmic fraction, saved for further protein analysis (Gholipour *et al.* 2010) .

Purification of recombinant enzyme

To purify the produced recombinant enzyme, the mixture of the culture medium and bacteria was first centrifuged and the supernatant liquid was kept at 80 °C for 20 minutes and was then centrifuged at 1000 rpm for 15 minutes for the bacterial proteins to precipitate. Amicon filters and dialysis bags were used to remove remaining bacterial proteins remaining in the supernatant liquid.

Measuring the activity of alpha-amylase enzyme

The DNS method was used to measure the activity of the alpha-amylase enzyme. In this method, 0.5 milliliter of the enzyme solution was placed beside 0.5 milliliter of the substrate (1% starch) and the reaction was stopped after three minutes by adding one milliliter of the reagent solution 3, 5-Dinitrosalicylic acid .The obtained solution was put in a water bath at

100 °C for five minutes and then, immediately, its temperature was lowered to 35 °C with the help of an ice bath. Ten milliliters of distilled water was then added to the solution and its degree of absorption at the wavelength of 450 nanometers against the distilled water control was read with the help of a spectrophotometer. To determine the quantity of reducing sugars in the solution containing the enzyme, one milliliter of the DNS solution was added to 0.5 milliliter of the enzyme solution, 0.5 milliliter of 1% starch was then added to this solution, and, after adding distilled water, the degree of absorption at 540 nanometers was read following the above-mentioned procedure. This obtained degree of absorption was deducted from the main one to determine the degree of absorption only related to enzymatic activity. The obtained figure was put in the equation for the standard curve without applying any coefficients, and the following relation was used to calculate enzymatic activity (Sajedi *et al.* 2004), (Miller 1959).

Enzymatic activity =

$$\frac{\text{Number of micromoles of maltose obtained from the equation for the standard curve}}{\text{Time (three minutes)}}$$

Results

Polymerase chain reaction

The Pfu polymerase enzyme was used for the correct replication of the studied sequences. This enzyme, due to its 3'-5' exonuclease activity, can replicate DNA with the lowest number of errors. The mean error rate of this enzyme in replication (1.3×10^{-6}) is about six times less than that of the Taq polymerase enzyme (which is 8×10^{-6}). (Cline *et al.* 1996). The polymerase reactions led to replication of a fragment with the length of 1472 base pairs. Figure 3-1 shows replication of this fragment using the PCR method (Figure 1).

Cloning of the fragment

In this research, the pET-26b (+) expression vector, which is one of the strongest known expression

vectors, was employed. The plasmid of this vector was extracted from the *E. coli* DH5 α bacteria. This plasmid was then digested and made linear with the addition of the *Xoh1* and *Msc1* enzymes.

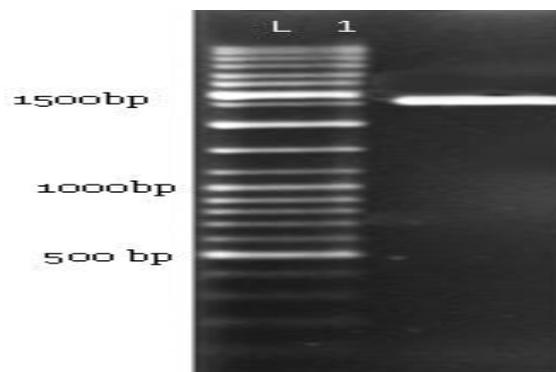


Fig. 1. Replication of the alpha-amylase gene using the Pfu polymerase enzyme, L: 100 bp plus Ladder, 1: 1472 bp alpha-amylase.

The MC1061 strain of the *E. coli* bacteria was used to transfer and replicate fragments that were produced in the cloning reaction. This strain has great ability in becoming competent, in accepting fragments, and in creating recombinant colonies. Naturally, bacteria that grew in the presence of kanamycin had the pET plasmid, but the possibility of rejoining made it necessary to figure out a way for identifying bacteria that contained the fragment. The PCR method was used to achieve this goal. These reactions took place by using the forward and reverse primers of the T7 vector. After making sure that the suitable fragment was present in the selected bacteria, the fragments were sequenced with successful results (Figure 2).

Expression of the recombinant alpha-amylase enzyme

The expression bacteria *E. coli* BL21 (DE3) was used to achieve abundant expression of the vectors containing the suitable fragment. After preparing BL21 (DE3) bacteria that had been made competent, the plasmid containing the desired fragment was extracted from the MC1061 bacteria and transferred to the competent BL21 (DE3) bacteria. A liquid culture medium, the TB culture medium that contained greater quantities of various nutrients including carbon sources, was used for the abundant expression of the recombinant proteins. Various

studies have shown that separating the bacterial growth phase from the protein production phase increases efficiency of expression (Choi *et al.* 2006). This point was also taken into consideration in this research by culturing the bacteria first in the TB culture medium and, only after the light absorption coefficient (λ_{600nm}) had reached 1.5-2 and the bacteria had grown sufficiently, was the isopropyl β -D-1-thiogalactopyranoside (IPTG) used for the expression of the recombinant protein. The pH of the culture medium was raised to about seven, or a little higher, by using 10 N KOH when necessary. The 12 percent SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was used to study the expression of the desired proteins. Figure 3-3 shows the protein profiles of the electrophoresed samples. Two groups of controls were employed to study the expression of the transgenic proteins. The first control group consisted of bacteria containing the desired fragment to which IPTG had not been added. This group of bacteria grew as the non-induced sample beside the induced ones. The presence of a protein band in the induced samples, and its absence in the non-induced ones, indicated the expression of the recombinant protein. The second control group consisted of the expression bacteria containing the pET-26b (+) vector that lacked any type of foreign genes. Comparison of the protein profiles of the two control groups with the induced transgenic bacteria indicated that transgenic proteins had been produced. Moreover, the recombinant protein band was observed both in the preplasmic and in the cytoplasmic spaces (Figure 3).

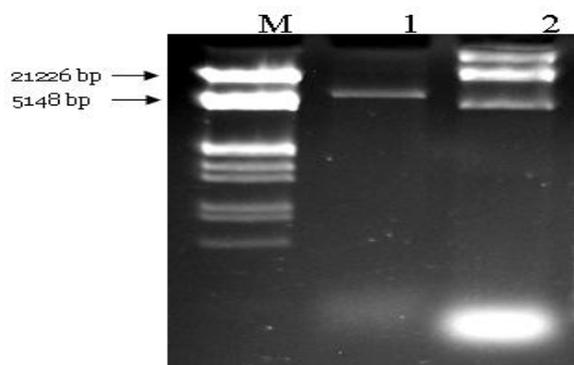


Fig. 2. Enzymatic digestion of pET-26b (+) with *XhoI* and *MscI* enzymes, M: molecular marker III, 1: Digested plasmid, 2: Non digested plasmid.

Activity of the alpha-amylase enzyme

The DNS method was used to measure the activity of the alpha-amylase enzyme. A 10-milligram per milliliter stock solution of maltose and 0.1, 0.5, 1, 3.5, and 4 dilutions of it were prepared. One milliliter of the DNS solution was added to one milliliter of each of these maltose concentrations, which were put in a boiling water bath for five minutes and, then, were immediately cooled to room temperature with the help of an ice bath. Light absorption was then read against the control (one milliliter of distilled water + one milliliter of the DNS solution).

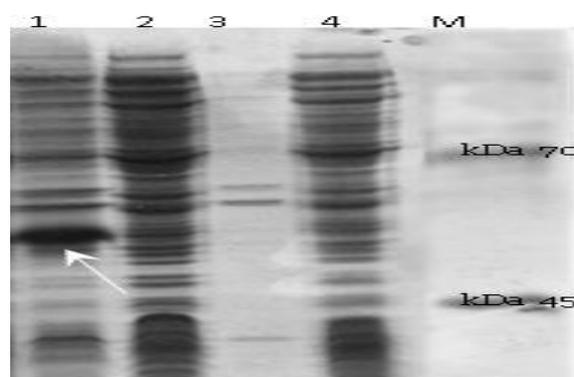


Fig. 3. Alpha-amylase recombinant protein expression in *E. coli* bacteria M: indicates proteins, 1: induced alpha-amylase, 2: non-induced alpha-amylase, 3: induced pET vector, 4: non-induced pET vector. The white arrow indicates the produced recombinant protein.

Discussion

The genus *Bacillus* produces a large variety of extracellular enzymes, some of which are of industrial importance, e.g., neutral proteases and α -amylases. It has commonly been reported that expression of α -amylase in various *Bacillus* species, including *B. licheniformis*, is temporally regulated, in that it is expressed predominantly after the exponential growth phase (Priest 1977). Because of the importance of the alpha-amylase enzyme in various industries, and due to the low level of the expression of this enzyme in various organisms, we extracted the gene related to this enzyme in *B. licheniformis* and transferred it to the expression vector pET-26 b (+). This is one of the strongest known expression vectors and can secrete the recombinant protein to the

periplasmic space. The recombinant vector was then transferred to the *E. coli* BL 21 bacteria. The periplasmic expression causes some post translational modification events which cannot be done in cytoplasmic expression. In addition, secretion of recombinant proteins to the periplasm of *E. coli* has several advantages over intracellular production among which are separation from cytoplasmic proteins, enhanced biological activity, enhanced product solubility and the ease of protein purification. We have successfully transferred and expressed an alpha-amylase gene in *E. coli* BL21 (DE3). Expression of foreign gene into *E. coli* BL21 was confirmed by SDS-PAGE analysis. SDS-PAGE analysis showed that recombinant enzyme was produced in inclusion body. In this study we didn't consider His tag sequences for purification of recombinant alpha-amylase. Purification was used by heat treatment and dialysis method. Heat treatment caused to solve inclusion body and activate enzyme. The enzyme activity was performed by Dinitro Salicylic Acid technique. In similar study, expression of alpha-amylase in *Bacillus licheniformis* was done. They showed that a-amylase production was related to the nature of the carbon source used for growth. If glucose was present in ample amounts, α -amylase was repressed, even if starch or other carbon sources were also present (Rothstein *et al.* 1986). In another investigation, the structural gene was cloned for extracellular α -amylase from a thermophile, *B. stearothermophilus* into vector plasmids pTB90 and pTB53. The gene was expressed in both *B. stearothermophilus* and *B. subtilis*. However, the level of gene expression in *B. subtilis* was lower than that in *B. stearothermophilus* (Aiba *et al.* 1983). Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes was studied in another survey. Amylase activity was maximum in the temperature range of 50° - 70°C, whereas this temperature range was deleterious for this bacteria. Also maximum enzyme activity was observed at 2% of starch concentration (Mishra and Behera 2008).

Conclusions

In general, the studied properties of the native α -

amylase from *B. licheniformis* (PTCC 5027) make it a good candidate for wide industrial applications; as additives in laundry detergents, and starch modification.

Knowledgments

The authors would like to thank Research Deputy of Microbiology department, Islamic Azad University of Kerman, Iran for financial supports and valuable contribution.

References

Aiba S, Kitai K, Imanaka T. 1983. Cloning and expression of thermostable α -amylase gene from *Bacillus stearothermophilus* in *Bacillus stearothermophilus* and *Bacillus subtilis*. Applied and Environmental Microbiology **46**, 1059-1065.

Alariya S, Sethi S, Gupta S, Gupta B. 2013. Amylase activity of a starch degrading bacteria isolated from soil. Archives of Applied Science Research **5**, 15-24.

Choi J, Keum K, Lee S. 2006. Production of recombinant proteins by high cell density culture of *Escherichia coli*. Chemical Engineering Science **61**, 876-885.

<http://dx.doi.org/10.1016/j.ces.2005.03.031>

Cline J, Braman J, Hogrefe H. 1996. PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. Nucleic Acids Research **24**, 3546-3551.

<http://dx.doi.org/10.1093/nar/24.18.3546>

Declerck N, Machius M, Joyet P, Wiegand G, Huber R, Gaillardin C. 2002. Engineering the thermostability of *Bacillus licheniformis* α -amylase. Biologia, Bratislava **57**, 203-211.

Dewaliya V, Jasodani R. 2013. Isolation and identification of *Bacillus licheniformis* for biosurfactant production. Journal of Microbiology **2**, 14-19.

Gholipour A, Moosavian M, Galehdari H,

- Makvandi M, Memari H, Alvandi A.** 2010. Cloning and periplasmic expression of peptidoglycan-associated lipoprotein (pal) protein of *Legionella pneumophila* in *Escherichia coli*. *Jundishapur Journal of Microbiology* **3**, 1-9.
- Gupta R, Gigras P, Mohapatra H, Goswami V, Chauhan B.** 2002. Microbial alpha amylase: A biotechnological perspective. *Process Biochemistry* **38**, 1599-1616.
[http://dx.doi.org/10.1016/S0032-9592\(03\)00053-0](http://dx.doi.org/10.1016/S0032-9592(03)00053-0)
- Kuriki, Imanaka T.** 1999. The concept of the alpha amylase family: Structural similarity and common catalytic mechanism. *Journal of Bioscience and Bioengineering* **87**, 557-565.
[http://dx.doi.org/10.16/S1389-1723\(99\)80114-5](http://dx.doi.org/10.16/S1389-1723(99)80114-5)
- Lonsane B, Ramesh M.** 1990. Production of bacterial thermostable alpha-amylase by solid-state fermentation: A potential tool for achieving economy in enzyme production and starch hydrolysis. *Advances in Applied Microbiology* **35**, 1-56.
- Maarel V, Marc J.** 2002. Properties and applications of starch converting enzymes of the alpha amylase family. *Journal of Biotechnology* **94**, 137-155.
[http://dx.doi.org/10.1016/S0168-1656\(01\)00407-2](http://dx.doi.org/10.1016/S0168-1656(01)00407-2)
- Miller G.** 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* **31**, 426-428.
<http://dx.doi.org/10.1021/ac60147a030>
- Mishra S, Behera N.** 2008. African journal of biotechnology. Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes **7**, 3326-3331.
- Ozcan N, Altinalan A, Ekuncu M.** 2001. Molecular cloning of an a-amylase gene from *Bacillus subtilis* rskk246 and its expression in *Escherichia coli* and in *Bacillus subtilis*. *Turkish Journal of Veterinary and Animal Sciences* **25**, 197-201.
- Pandey A, Nigam P, Soccol C, Soccol V, Singh D, mohan R.** 2000. Advances in microbial amylases. *Biotechnology and Applied Biochemistry* **31**, 135-152
- Priest F.** 1977. Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriological Reviews* **41**, 711-753.
- Rana N, Walia A, Gaur A.** 2013. α -amylases from microbial sources and its potential applications in various industries. *National Academy Science Letters* **36**, 9-17.
<http://dx.doi.org/10.1007/s40009-012-0104-0>
- Reddy N, Nimmagadda A, Rao K.** 2003. An overview of the microbial alpha amylase family. *African Journal of Biotechnology* **2**, 645-648.
- Rothstein D, Devlin P, Cate R.** 1986. Expression of alpha-amylase in *Bacillus licheniformis*. *Journal of Bacteriology* **168**, 839-842.
- Roy J, Mukherjee A.** 2013. Applications of a high maltose forming, thermo-stable α -amylase from an extremely alkalophilic *Bacillus licheniformis* strain AS08E in food and laundry detergent industries. *Biochemical Engineering Journal* **77**, 220-230.
- Sajedi R, Naderi-Manesh H, Khajeh K, Ahmadvand R, Ranjbar B, Asoodeh A.** 2004. A ca-independent alpha amylase that is active and stable at low pH from the *Bacillus sp.* KR-8104. *Enzyme and Microbial Technology* **36**, 666-671.
<http://dx.doi.org/10.1016/j.bej.201306.012>
- Sarbatly R, England R.** 2004. Critical review of membrane bioreactor system used for continuous production of hydrolyzed starch. *Chemical and Biochemical Engineering Quarterly* **18**, 155-165.
- Sathyavathan P, Krithika S.** 2014. Production and optimization of protease from *Bacillus licheniformis* NRRL-NRS-1264 using cheap source substrates by submerged (SmF) and solid-state fermentation (SSF). *International Journal of*

ChemTech Research **6**, 286-292.

Sellami-Kamoun A, Haddar A, El-Hadj Ali N, Ghorbel-Frikha B, Kanoun S, Nasri M. 2008. Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in commercial solid laundry detergent formulations. Microbiological Research **163**, 299–306.

<http://dx.doi.org/10.1016/j.micres.2006.06.001>

Smitha S, Bhat S. 2013. Thermostable bacteriocin BL8 from *Bacillus licheniformis* isolated from marine sediment. Journal of Applied Microbiology **114**, 688–694.

<http://dx.doi.org/10.1111/jam.12097>

Torry S. 1983. Enzyme technology. Noyes publications USA, 33-63.

Tucker GA, Woods L. 1995. Enzyme in food processing. Blackie academic press UK, 190-255.

Universiteit O. 1993. Technological application of biocatalysts. Butterworth-Heineman UK, 20-50.

Vinhhinen M, Mantsala P. 1989. Microbial amyolytic enzymes. Critical Reviews in Biochemistry and Molecular Biology **24**, 329-363.

<http://dx.doi.org/10.3109/10409238909082556>

Windish W, Mhatre N, Wayne W. 1965. Microbial amylases, In : Advances in applied microbiology. Academic Press, New York, NY, USA, :273–304.