An Acad Bras Cienc (2022) 94(4): e20200913 DOI 10.1590/0001-3765202220200913
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

CELLULAR AND MOLECULAR BIOLOGY

Cloning and Expression of β-(1,3-1,4) Glucanase (Lichenase) Gene in *Bacillus subtilis* RSKK246 to create new Probiotic in aquaculture

ÖZGEN A. ÇAM, MAKBULE BAYLAN & GAMZE MAZI

Abstract: β-Glucan is an essential component of the cell walls of grains such as oats and barley. 1,3-1,4-β-D-glucan 4-glucanhydrolase (β-glucanase or lichenase) (EC 3.2.1.73) is an enzyme with the ability to hydrolyze β-glucans. In this research, β-Glucan which is a good source of feed additive fish probiotics, was used in order to benefit from feed quality in fishery products, to increase live weight gain and to strengthen the immune system. In this study, recombinant vector pNW33N carrying the β-(1,3-1,4) glucanase (lichenase) gene of *Streptococcus bovis* genome was transferred to *Bacillus subtilis* RSKK246 (CMCase+) strain by electroporation. Subsequently, electrotransformation was performed on LB-agar plates containing lichenan and enzymatic activity regions of recombinant *B. subtilis* RSKK246 colonies were observed by staining with Congo red. In addition, the DNA from the recombinant plasmid pNW33N+Lichenase (pNW33NLic) was cut on both the BamHI and HindIII endonucleases and observed on the lichenase gene (1800 bp) agarose gel. On the other hand, the protein band corresponding to 26 kDa of the recombinant enzyme was observed by zymogram analysis. These results indicate that the β-(1,3-1,4) glucanase gene has been successfully expressed to the *B. subtilis* strain RSKK246.

Key words: *Bacillus subtilis,* β (1,3-1,4) glucanase, gene transfer, probiotic.

INTRODUCTİON

Together with the increasing human population, the need for quality protein sources increases in parallel. Sources of animal protein; while fishery products, red meat, white meat, eggs and milk are formed among them, ease of digestion of fishery products, rich in essential fatty acids and cardiovascular health and positive effects on brain development are more prominent than other protein sources. However, nutritional habits vary according to the level of development of countries, geographical structure and traditions and income levels of the regions where the societies are located. Turkey is a developing country, with more plant-based foods to meet protein needs. Annual consumption of 25 kilograms of fish found in European countries, while reaching an even higher figure in the Far East countries, annual fish consumption per capita in Turkey is only 8 kilograms (Tüik 2015). In addition to being surrounded by seas, our country is also fortunate in terms of rich in the biodiversity and consumable aquaculture products. However, despite the increase in our aquaculture production every year, there is not enough demand in the domestic market. As in the other field of animal production, practices are carried out to increase the yield and quality of aquaculture. The main reason behind these practices is due to the increasing demand for animal food due to the ecological conditions that are advancing in parallel with the increasing world population. The most common of these

is to provide functionality by adding different properties to the feeds.

These manipulations have a positive effect on yield, either directly or indirectly, either by facilitating the maintenance of the feed or by increasing the digestibility or by supporting the animal's immune system.

The paper aims to transfer and express of β-(1,3-1,4)-glucanase gene into CMCase producing *Bacillus* sp to create a recombinant probiotic for aquaculture by using biotechnological methods and genetic engineering techniques. Thus, new recombinant probiotics have been developed that produce feed-improving enzymes.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth media

Bacillus subtilis RSKK246 and pNW33N+lichenase (pNW33NLic) shuttle vector to be used in this study is present in Animal Biotechnology and Genetic Engineering Laboratory (from Cukurova University, Faculty of Agriculture Zootechni Department). *B. subtilis* RSKK246 were maintained in medium (10% v/v glycerol). *B. subtilis* RSKK246 and *Bacillus sp.*/pNW33NLic cells were cultured in LB (1% w/v Bacto tryptone, 0.5% w / v NaCl, 1.5% agar yeast extract, pH 7.5) at 37ºC. The solid medium was made by adding 1.5% agar to the LB liquid medium. Growth media were supplemented with 30 µg/mL Chloramphenicol (Cml) to select recombinant *B. subtilis* RSK246 cells.

Isolation, structure of pNW33NLic plasmid

Standard molecular biology protocols (Hardy 1985, Sambrook 1989) were used for plasmid isolation and DNA manipulation, unless otherwise explicitly described. Recombinant pNW33NLic plasmid DNA from the *Bacillus sp.* colony was isolated according to Hardy (1985). *Bacillus* sp./pNW33N+lic (Figure 1) was linearized by HindIII and BamHI enzymes for control

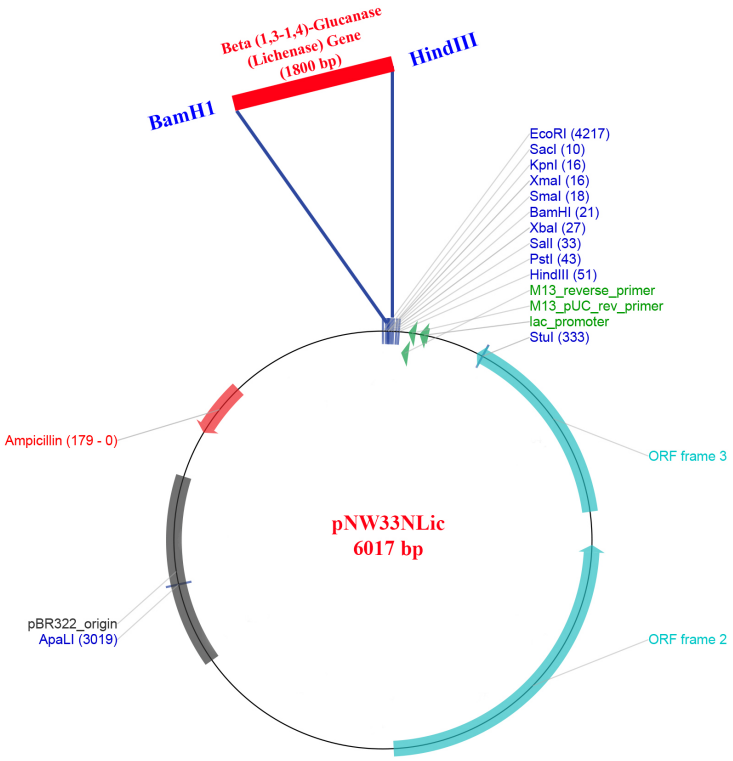

Figure 1. Structure of pNW33NLic plasmid (pNW33N (4217 bp) plus lichenase gene (1800 bp).

purposes and then electrophoresed on a 0.8% agarose gel and visualized.

Bacillus subtilis RSKK246 bacteria were plated on Petri plates containing LB/Agar/CMC and incubated overnight at 37° C. The next day, the phenotypic determination of CMCase was performed with Congo red staining of bacteria. After overnight growth, the petri plates were flooded with a 0.2% (w/v) Congo red solution. After 15 minutes of incubation at room temperature, the Congo red solution was replaced with a 1 M NaCl solution for 30 minutes. The NaCl solution was removed.

Transformation of *Bacillus subtilis* RSKK246 by electroporation

The electroporation procedure to transfer of the recombinant pNW33NLic into *B. subtilis* RSKK246 was used by the protocol described by Xue et al. (1999) in the present study. For electroporation, *B. subtilis* RSKK246 cells were grown in LB supplemented with 0.5 M sorbitol at 37ºC to OD600nm 0,85-0,95. Cells were recovered by centrifugation, washed four times in cold SHMG electroporation buffer (0.25 M sucrose, 1 mM HEPES, 1 mM MgCl2, 10% (v/v) glycerol, pH 7.0), and resuspended in the buffer in 1/40 of the original cultivation volume. For transformation, 60 µl of the cells were incubated with plasmid DNA (0.5 µg) in a sample cuvette (Bio-Rad) with an interelectrode distance of 0.1 cm on ice for 2-3 minutes and pulsed by an Invitrogen ElectroporatorII apparatus set at 25µF, 200 Ω and 16.5 kV/cm. The electric shocking cuvettes were taken back on ice. Bacteria in the electroporation cuvette were separated into 1 ml liquid LB medium containing 0.5 M sorbitol and incubated at 37 ºC for 3 hours. Thus, the antibiotic resistance gene in the bacteria that have received the plasmid is expressed and the adaptation of the bacteria to the antibiotic medium is provided. Transformants with pNW33NLic were selected on LB/Lichenan/Agar plates containing 30 µg/ml Chloramphenicol (Cml) at 37ºC. At the end of the period, the bacteria were plated on LB/Lichenan/Agar/Cml (30 µg mL^{-1} chloramphenicol) plates containing antibiotics. Plates were incubated overnight at 37 ºC. Controls were plated on LB/Lichenan/Agar plates with and without antibiotics, and were incubated overnight at 37 °C.

Detection of lichenase activity of recombinant *Bacillus sp.*/pNW33NLic colonies on lichenan plates

Lichenase positive transformants were detected using LB plates containing agar, 30 µg/mL^{-1} Cml and 0.1% (w/v) lichenan. After growing at 37°C overnight, 0.1% (w / v) Congo red solution was added to the Petri plates. After standing at room temperature for 15 minutes, the Congo red solution was changed with 1 M NaCl solution. Then, the NaCl solution was drained and bright areas appeared around the positive colonies.

Determining of lichenase enzyme on SDS-PAGE and SDS-Lichenan-PAGE

To detect of lichenase enzyme secreted by *Bacillus subtilis* RSKK246 and recombinant *Bacillus subtilis* RSKK246 strains, the sonicated cell extract was centrifuged at 4500 rpm for 10 minutes to remove cell or cell debris. The extracellular extracts (supernatants) were mixed with 1:1 volume of 20% w/v TCA for precipitation. Total proteins were collected by re-centrifugation.

Protein analysis was performed by using a denaturating polyacrylamide gel electrophoresis (SDS-PAGE, 12% w/v). Zymogram analysis was done SDS-lichenan-PAGE. SDS-lichenan-PAGE (0.2% w/v) were done as described with slab gels (12% wt/v acrylamide) according to Laemmli (1970). After the electrophoresis, the gel was stained for 1 h with Coomassie blue R 250 dye in

methanol–acetic acid–water solution (4:1:5) and destained in the same solution without dye.

The gel was then transferred into a renaturation solution (50 mM sodium phosphate buffer, 5 mM β-mercaptoethanol, 1 mM EDTA) and incubated overnight at 4°C. Renaturation of enzyme proteins was carried out by keeping the gel overnight in a solution containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), 5 mM β-mercaptoethanol and 1 mM EDTA at 4°C.The gel was then transferred onto a glass plate, covered with a film, and incubated at 30°C for 4 h. The gel was stained in a solution of Congo red (0.1% w/v Congo-red, 0.2 M NaOH), for 1 h, and destained in 1 M NaCl for 30 min. Clear bands indicated the presence of β-(1,3-1,4) glucanase (Lichenase) activity.

RESULTS

In this study, recombinant pNW33NLic plasmid for control was isolated from *E. coli* cells. They were; then subjected to restriction fragment length analysis together with PCR amplified DNA fragment on agarose gel electrophoresis (0.8% w/v) encoding the gene. Lichenase gene fragment (~1.8 kbp) amplified by PCR and restriction endonuclease digested recombinant plasmids confirmed the success of the cloning experiments. Recombinant plasmid digested with HindIII and BamHI was yielded the same DNA fragments consisting of pNW33N and β-(1,3-1,4)glucanase (lichenase) gene (Figure 2).

Determination of CMCaz activities o*f Bacillus subtilis* RSKK246 bacteria

Bacillus subtilis RSKK246 bacteria were planted on Petri plates containing LB/Agar/CMC and incubated overnight at 37° C. The next day, the phenotypic determination of CMCase was performed with Congo red staining of bacteria. As a result of the processes, bright yellow zones were seen around positive colonies (Figure 3a,b).

pNW33NLic carrying lichenase gene (Figure 1) was then introduced by electrotransformation

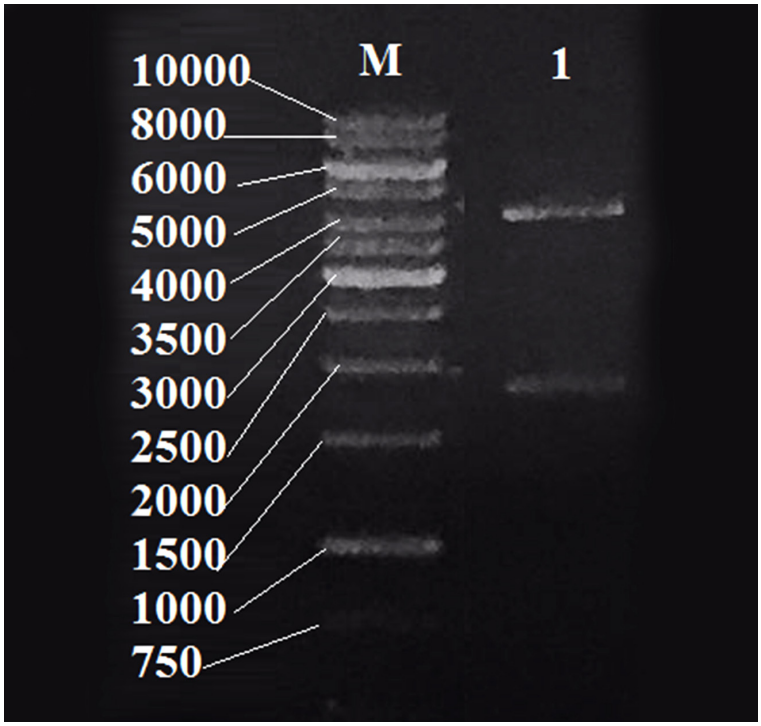

Figure 2. Insert and PCR analysis of *E. coli*/ pNW33NLic (M: 1 kbp DNA markers, 1- PCR amplified fragment of lichenase gene from pNW33NLic, 2- pNW33NLic /*HindII and BamHI.*

into *B. subtilis* RSKK246. The colonies were observed on LB agar medium with and without Cml for comparative phenotypic testing. To reveal the recombinant colonies, both the non-recombinant colonies and the recombinant colonies grew on the solid LB medium after transformation were reinoculated on the LB agar medium added Cml. Recombinant *B. subtilis* RSKK246 developed and colonized plaques containing antibiotics. It was observed that the original *B. subtilis* RSKK246 did not form colonies in antibiotic-containing plates (Figure 4a,b).

Culture supernatants of recombinant *B. subtilis*/pNW33NLic and non-recombinant *B. subtilis* RSKK246 strains were applied to SDS-PAGE and SDS-Lichenan-PAGE to visualize total proteins and zymogram analysis, respectively. For zymogram analysis, denaturated proteins were renatured on SDS-Lichenase-PAGE after removing denaturating agents from the gel and then allowing the enzyme to digest substrate, thereby producing clear zones on the gel. On zymogram analysis, only β-(1,3-1.4)-glucanase (lichenase) protein band of *B. subtilis* with 26 kDa in size was showed a clear zone together with intracellular protein counterparts of all other recombinant *B. subtilis* RSKK246 strains (Figure 5).

DISCUSSION

The increase in the human population and the arbitrary use of antibiotics exert significant pressure on agriculture, animal husbandry and aquaculture. Efforts to develop antibiotic-resistant pathogenic strains have accelerated, with the use of antibiotics restricted by the authorities. In seafood systems, probiotic bacteria are used to prevent pathogenic developments. In this sense, *B. subtilis* is an ideal multifunctional probiotic bacteria capable of solving these problems and increasing the profitability of aquaculture. *Bacillus subtilis* C-3102 has been used as a direct-fed microbial or probiotic product since 1986 to improve production performance in broilers worldwide (Jeong & Kim 2014). By adding enzymatic mixtures containing bacterial glucanases to animal feeds, especially broiler and pig feeds, the digestibility of barley-based feeds increases and health

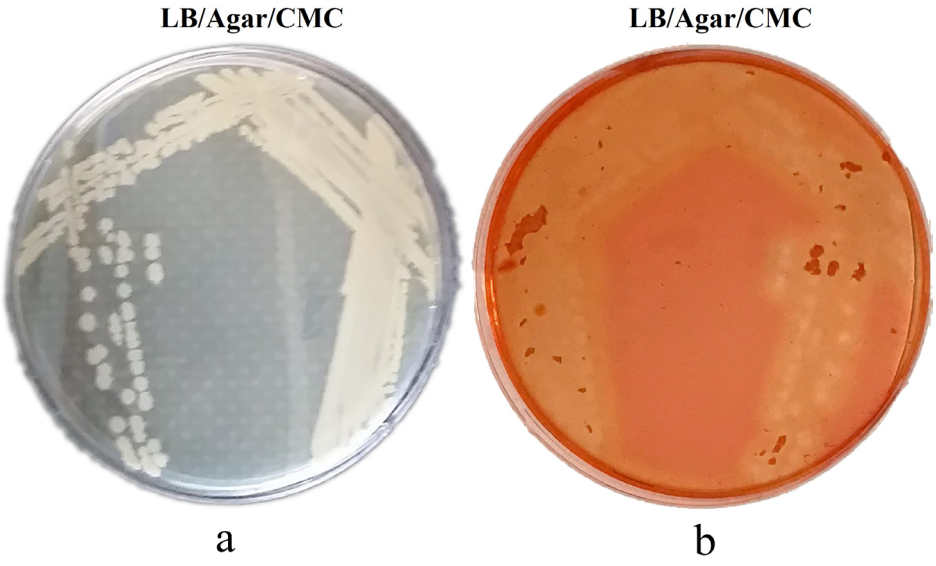

Figure 3. ***Bacillus subtilis*** **RSKK246 colonies (a) and CMCase phenotypic testing via Congo red dying (b) on LB/Agar/CMC plate.**

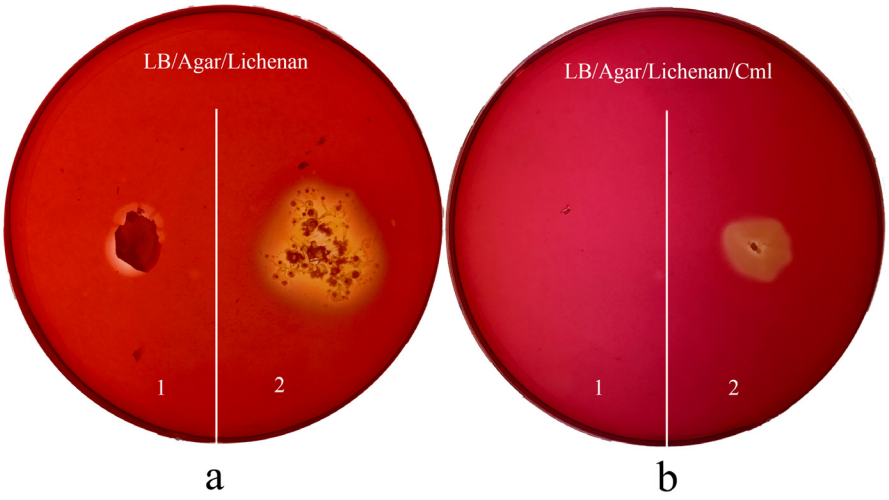

Figure 4. Comparative phenotypic test of recombinant *B. subtilis* RSKK246/pNW33NLic and original *B. subtilis* RSKK246 bacteria 1: *B. subtilis* RSKK246 2: *B. subtilis* RSKK246/pNW33NLic (a: Antibiotic-free substrate plaque b: substrate plate containing antibiotics).

problems (sticky dropping) are reduced (Stone & Clarke 1992, White et al. 1983). In addition, lichenase and phytase enzyme genes were cloned in *B. coagulans* to develop probiotics for poultry (Aşan Özüsağlam 2007, Aşan Özüsağlam & Özcan 2009)

The lichenase gene, which is very attractive for increasing the digestibility of fish and other animal feeds, increasing feed utilization rate and strengthening the immune system and increasing live weight, has been transferred to different bacteria by using many cloning studies.

In previous studies, the bacterial lichenase genes were transferred into various microorganisms such as *E.coli* (Louw & Watson 1993, Spilliaert et al. 1994, Chen et al. 1997, Kim et al. 2002, Gosalbes et al. 1991), *L. reuteri* (Heng et al. 1997), *B. subtilis* (Gosalbes et al. 1991), *S. bovis*, *L. lactis* and *E. feacalis bacteria* (Ekinci et al. 1997).

With this study, the recombinant pNW33NLic vector carrying β-(1,3-1,4) glucanase (Lichenase) gene belonging to the *Streptococcus bovis* genome was cloned in *Bacillus subtilis* RSKK246 strain by electroporation technique and expressed. The enzyme secreted from recombinant *B. subtilis* RSKK246 was found to be active, showing intracellularly clear zones on the LB-agar plate containing lichenan. On the other hand, zymogram analysis clearly indicated that activity bands surrounded with clear zones confirming the renaturation of the denatured enzyme.

Besides, as a result of zymogram analyzes, *B. subtilis* RSKK246 did not show lichenase activity, while *B. subtilis* RSKK246/pNW33NLik strain produced a 26 kDa molecular weight lichenase band. Both plaque, zymogram, and agarose gel analyzes were the most apparent indications that the lichenase gene was transferred to the *B. subtilis* RSKK246 bacteria with the pNW33N vector. The molecular weight of the lichenase gene was found to be 27 kDa (Chen et al. 1997), 29 kDa (Louw & Watson 1993, Gosalbes et al. 1991), 25 kDa (Ekinci et al. 1997) in many studies in which cloning studies were conducted. *B. subtilis* RSKK246/pNW33NLik strain supports our study to give a lichenase band with a molecular weight of 26 kDa.

In this study, the lichenase gene belonging to *S.bovis* was cloned for the first time in the strain of *B. subtilis* RSKK246. Bacillus strains are the most used bacteria in industrial enzyme production since they are capable of

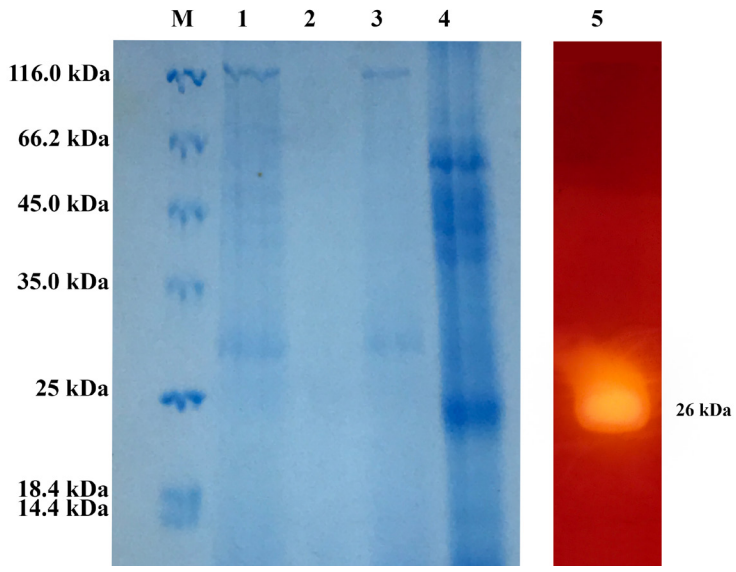

Figure 5. Comparition of total proteins of recombinant and non-recombinant strains on SDS-PAGE (1-4) and zymogram analysis of lichenase enzyme on SDS-Lichenan-PAGE (5).

secreting the enzymes they produce out of the cell. Bacillus, which are used in various biotechnological studies, have a commercial importance due to the proteins they produce, are not pathogens and form endospores are other important reasons for these bacteria to be preferred (Vehmaanperä 1989, Harwood 1992, Özcan 1996, Özcan & Özcan 2001). Also, Lichenase enzyme was preferred in this study due to reasons such as higher hydrolysis power of β-glucans and positive effects on the immune system compared to other glucanase types (Uhlig 1998).

As a result, experiments can be made by adding newly created recombinant bacteria (*B.subtilis* RSK246/pNW33NLic) as a probiotic feed additive to the rations of fish and other farm animals. With this enzyme developed in this way, it can be tested how much the animals strengthen the immune system. It can also be used as a food supplement for animal feed and other biotechnological applications. On the other hand, genes can be cloned in different bacteria to produce more abundantly, purer and more economically.

Acknowledgments

The authors are grateful to the Scientific Research Projects Commission of Cukurova University (Project number: FYL-2016-5889) for funding this program. This article was produced from Özgen Arzu ÇAM's thesis.

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How to cite

ÇAM ÖA, BAYLAN M & MAZI G. 2022. Cloning and Expression of β-(1,3-1,4) Glucanase (Lichenase) Gene in *Bacillus subtilis* RSKK246 to create new Probiotic in aquaculture. An Acad Bras Cienc 94: e20200913. DOI 10.1590/0001-3765202220200913.

Manuscript received on June 11, 2020;
accepted for publication on December 4, 2020

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Ö.A.Ç. and G.M.: performed the growth of bacterial isolates, cloning studies, detection of lichenase activity; M.B.: coordinated the study and wrote the manuscript; all authors commented on and improved the manuscript.