JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

CLONING, SEQUENCING AND IN SILICO ANALYSIS OF AN AMYLASE OF STREPTOCOCCUS LUTETIENSIS RB4 ISOLATED FROM BOVINE RUMEN

Dilek Ozgun Ekiz [1], *Ugur Comlekcioglu* [2], *Ashabil Aygan**[1]

Address(es): Ashabil Aygan,
[1]Kahramanmaras Sutcu Imam University, Faculty of Arts and Sciences, Department of Biology, 46040, Kahramanmaras, Turkey, phone number: +90 344 300 1405.
[2]Ghent University, Faculty of Veterinary Medicine, Department of Reproduction, Obstetrics and Herd Health, 9820, Merelbeke, Belgium.

*Corresponding author: ashabil@ksu.edu.tr

https://doi.org/10.55251/jmbfs.5940

ARTICLE INFO

Received 7. 3. 2022
Revised 28. 6. 2022
Accepted 13. 9. 2022
Published 1. 12. 2022

Regular article

OPEN ACCESS

ABSTRACT

Streptococcus lutetiensis RB4 was isolated from the rumen of beef cattle. An amylase gene from the isolate RB4 was cloned and expressed in *Escherichia coli* DH5α by using pGEMT-Easy vector system. *S. lutetiensis* is a member of *S. bovis* / *S. equinus* complex. Sequence characterization of the cloned gene *AmySL* displayed a 1640 bp long with an open reading frame coding 298 amino acids. AmySL has a molecular mass of 33.456 Da which was considerably smaller. The optimal pH and temparature for *AmySL* werepH 6.5 and 40ºC, respectively. AmySL hydrolyzed starch to maltose and other oligosaccarides as the final products. Random coils (37.25%) and α-helix (34.23%) were dominated the secondary structure of AmySL. *S. lutetiensis* RB4 grouped together with *S. macedanicus* and *S. pasterurianus* according to phylogenetics analysis. This research identified some features of the amylase gene isolated from *S. lutetiensis*. Although *S. bovis*, which has an important place in amylolytic bacteria, is known to be an important factor in ruminal acidosis, the number of studies on the amylases of the SBSEC group is surprisingly low. Therefore, this study will add to our understanding of the enzymes belonging to the SBSEC complex involved in the breakdown of rumen starch.

Keywords: Amylase, Glycosyl hydrolase family 13, Phylogenetics, *Streptococcus*

INTRODUCTION

Ruminant animals provide their energy and essential nutrients from lignocellulosic plant materials through a complex symbiotic relationship with the microbiome they carry in their rumen **(Matthews *et al.*, 2019)**. Rumen microbiome composition is dynamically influenced by the feed type consumed by the host ruminant, and variations of the bacterial community in the rumen can fully affect the productivity and health of host ruminants, therefore, feeding ruminants a balanced diet is essential **(Amaro *et al.*, 2020; Lee *et al.*, 2019)**. However, in order to increase the capableness of livestock ruminants, a high feed diet is replaced with a high concentrated diet which increases levels of non-structural carbohydrates, and this switch often modifies the rumen ecosystem and increase the amount of lactic acid producers like *S. bovis* and *Lactobacillus* spp. **(Lee *et al.*, 2019)**. In ruminants, *S. bovis* is estimated to give 10^6 - 10^7 cells per milliliter of rumen ingredients **(Hudson *et al.*, 2000)** and reasoned one of the important contributory agents related to acute ruminal acidosis. *S. bovis* can grow rapidly with enough non-fibrous carbohydrates and produce a substantial volume of lactate and capsular polysaccharides resulting in acute rumen acidosis and bloat, respectively **(Herrera *et al.*, 2009)**.
The taxonomic place of *S. bovis* has been appeared in progress due to the development of molecular techniques **(Yu *et al.*, 2021)**, and *S. bovis*, synonymized with *S. equinus* and recently identified as *S. bovis* / *S. equinus* complex (SBSEC), known as non-enterococcal group D *Streptococcus* spp. **(Pompilio *et al.*, 2019)**. SBSEC group is complex and complicated due to contradicting classical analysis based on phenotypic features and contemporary disputes about molecular approaches for species-level identification **(Dekker *et al.*, 2016)**. Previously, SBSEC was divided into 3 biotypes designated as biotype I, biotype II/1 and biotype II/2 **(Chen *et al.*, 2021)**. Furthermore, SBSEC taxonomy has been revised based on genetic biomarkers, and SBSEC was defined as a group of seven species or subspecies, including *S. equinus*, *S. lutetiensis*, *S. infantarius* subsp. *infantarius*, *S. alactolyticus*, *S. gallolyticus* subsp. *pasteurianus*, *S. gallolyticus* subsp. *macedonicus*, and *S. gallolyticus* subsp. *gallolyticus* **(Park *et al.*, 2021)**.
Although *S. bovis*, which has an important place in amylolytic bacteria, is known to be an important factor in ruminal acidosis, the number of studies on the amylases of the SBSEC group is surprisingly low. For this reason, in this study, the amylase gene of an isolate belonging to the SBSEC group, which we isolated from the rumen content, was cloned, and characterized. According to the 16S rRNA analysis of this isolate, it was seen that the isolate belonged to the *S. lutetiensis* - *S. infantarius* group. The amylase gene of these species has not been characterized by cloning before. In this respect, the amylase gene belonging to this microorganism group was cloned for the first time in this study. The amylase gene characterized in this study differs from other *S. bovis* amylases with its low molecular weight (MW). Characterizing the amylase genes of these species may help develop important strategies against rumen acidosis.

MATERIAL AND METHODS

Sampling and bacterial isolation

The rumen content samples were obtained directly from a rumen of a slaughtered cow in Kahramanmaras Slaughterhouse. Samples were placed into sterile Hungate tubes with screw caps containing anaerobic culture media under refrigerated conditions. The samples were immediately transported to the laboratory in less than 1 h. Later, direct or diluted aliquots were spread onto Petri plates containing an anaerobic medium under strict anaerobic conditions, by using an anaerobic cabinet (Elektrotek AW200SG, West Yorkshire, United Kingdom). Anaerobic medium was formulated according to **Comlekcioglu *et al.*** (2008). Anaerobic medium contained starch (0.5%, w/v) as an energy source to stimulate the growth of starch degrading bacteria. The plates were then incubated at 40ºC under anaerobic conditions for 24-48 h. A pool of approximately 10 gram positive, catalase negative and starch degrading bacteria were selected and tested for their amylase producing capabilities, and finally, the isolate RB4 was selected for further experiments. The purified bacterial isolate RB4 was identified by sequencing the 16S rRNA. The universal primers used to amplify 16S rRNA were 27F and 1492R. RB4 was stored at -80°C with 60% sterile glycerol for use in further studies.

Cloning and expression of α-amylase gene

After genomic DNA isolation from the RB4 strain, amylase encoding gene amplification was accomplished using the primers AmySbF (5'-CTTTTTATGGTGGTGAATGG-3') and AmySbR (5'-TATCAAATGCAAACAGCACAA-3'). For primer designing, full-length sequence of amylases from *S. bovis* (Accession numbers AB000830.1 and U04956.1) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov). The primers were designed to cover the complete Open Reading Frame (ORF) of these

amylases and were synthesized by Ella Biotech GmbH, Germany. The amplification of the amylase gene was carried out by Polymerase Chain Reaction (PCR). After an initial denaturation step at 94°C for 5 min, the reaction mixture was allowed to run for 35 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C. The amplified PCR product was detected by 1% agarose gel electrophoresis and ligated in pGEM®-T Easy Vector System I (Promega). The recombinant plasmid, named as pGEMTA1 (containing amylase insert), was transformed to expression host *E. coli* DH5α using the $CaCl_2$ method to express the xylanase proteins. Transformed *E. coli* DH5α cells were spread onto Luria-Bertani (LB) agar plates containing ampicillin (50 µg/ml). Recombinant strains were screened for amylase activity by overlaying of 0.5% starch and 0.4% agarose in 50 mM sodium phosphate (pH 6.5) buffer. After incubation at 37ºC for 4 hours, amylase activity was detected by Lugol staining **(Rasiah and Rehm, 2009)**. An amylase positive *E. coli* strain was purified (*E. coli* A1) and stored in 15% (v/v) glycerol at -20ºC. For expression analysis, *E. coli* A1 was cultivated in LB medium including 50 µg/mL of ampicillin at 37°C in a shaking (150 rpm) incubator. After incubation, cells were pelleted and washed with 50 mM sodium-phosphate buffer (pH 6.5) twice. Then the cells were disrupted using a ball mill (Retsch) and resuspended in 50 mM sodium phosphate buffer (pH 6.5). Then the cell debris was removed by centrifugation and the supernatant was stored at –20ºC for enzyme assays.

Enzyme assays

Amylase activity was assayed using the DNS (dinitrosalicylic acid) method of Miller **(Miller, 1959).** The amount of reducing sugar, resulting from the enzymatic hydrolysis of 0.5% soluble starch in 50 mM sodium phosphate buffer (pH 6.5) at 40ºC for 30 min, was detected by a UV-Visible spectrophotometer at 540 nm. One unit of amylase was determined as the amount of the enzyme to release 1 µmol of reducing sugar per min. The effect of pH on enzyme activity was determined at 40ºC and pH 3.5-9.0. For this purpose, some substrate-containing solutions were used, such as 50 mM Acetate buffer (pH 3.5 to 5.6), Phosphate buffer (pH 6.0 to 8.0), Tris-HCl buffer (pH 8.0 to 9.0), and Glycine-NaOH (pH 9.0-12.0). The temperature optimum of amylase activity was found out by incubating the reaction mixture at different temperatures from 5 to 60ºC. On the other hand, the pH stability of the amylase was studied by pre-incubating the enzyme with different buffers ranging from 4.5 to 9.5 at 40ºC for 15 min. And thermostability was also determined by pre-incubating the enzyme for 60 min at temperatures from 30 to 70ºC. After the pre-incubation step, the enzyme was chilled in an ice bath for 5 min and then residual activity was determined as described above. All standard assay procedures were conducted in triplicate and the mean values were taken.

Zymogram and thin layer chromatography (TLC) analysis

The enzyme sample and protein marker (Serva) were analyzed with SDS-PAGE. It was accomplished with 12.5% of a gel including 0.5% (w/v) soluble starch. Zymogram analysis was performed in accordance with **Liu *et al*. (2007).** To determine the amylase activity on the gel, the gel was kept in 1% Lugol for 10 minutes. In terms of the end product analysis, enzyme was allowed to react with the substrate at optimum temperature for 15, 30 and 60 min. The samples were subjected to silica gel plate (Silica Gel60, Merck). Then plate was developed by placing in a chamber containing a mobile phase, chloroform-acetic acid-distilled water (6:7:1, v/v/v). Spots were visualized by spraying the air-dried plate with aniline-diphenylamine-ortophosphoric acid (1:1:10, v/w/v) prior to heating it in an oven at 150°C.

Sequencing and *in silico* analysis of amylase

The cloned amylase gene was amplified from pGEMTA1 by PCR. The amplified samples were electrophoresed on 1% agarose gel, then purified from the gel using GF1 AmbiClean Kit (Vivantis, Malaysia) following the manufacturer's instruction. The amylase gene sequence was analyzed on both strands by a company (Macrogen, Korea). Sequence alignment and data analysis were carried out using the computer programs ChromasPro V1.7.7 and Clone Manager 5, respectively. Analysis of physiochemical properties and amino acids available in essential polypeptide chain of the amylase gene were done using the ExPASy-ProtParam tool (https://web.expasy.org/protparam/). The predicted protein solubility (Sol) is calculated by using Protein-Sol tool (https://protein-sol.manchester.ac.uk/). Putative conserved domains were detected in CDD-BLAST (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The secondary structure of retrieved enzymes was performed using SOPMA tool in prabi (https://npsa-prabi.ibcp.fr/) by calculating the number of α-helices, turn, extended strand, random coil. SWISS-Model (https://swissmodel.expasy.org/) was used for the prediction of 3D structures of all retrieved sequences. SAVES v6.0 web-server (https://saves.mbi.ucla.edu/) has been used to evaluate the 3D models obtained from the selected sequences by using ERRAT **(Colovos and Yeates, 1993)**, Verify 3D **(Lüthy *et al.*, 1992)** and PROCHECK **(Laskowski *et al*., 2006)**. Sequence data of glycosyl hydrolase family 13 (GH13) amylases were obtained from NCBI database (https://www.ncbi.nlm.nih.gov/), then these sequences were used to evaluate phylogenetic relationships with the amylase obtained in this study. GH13 catalitic domain containing 30 proteins was analyzed by aligning the amino acid sequences using MAFFT web software **(Katoh *et al*., 2017)**. The evolutionary history was implicated on the basis of the Neighbor-Joining method. The evolutionary distances were then calculated with the Maximum Composite Likelihood method. Evolutionary analyses were finally achieved with MEGA X.

RESULTS AND DISCUSSION

Identification of *S. lutetiensis*

After bacterial isolation from rumen content, 10 amylolytic bacterial colonies were screened for their amylase production on starch agar media. Out of 10 bacterial colonies, one isolate, designated as RB4, showed good zone of hydrolysis at 40°C and was taken for further studies. The 16S rDNA of the isolate RB4 was sequenced and 1388 bp of 16S rRNA was obtained in good quality. Verification of 16S rRNA sequence homology of RB4 was checked with the BLAST algorithm and BLAST results indicated that this isolate belonged to the *Streptococcus* genus. BLAST showed that the isolate RB4 has maximum homology with *S. lutetiensis* (99%). The 16S rRNA sequence from RB4 was compared with sequences available in the GenBank database by phylogenetic analysis (Figure 1). The phylogenetic analysis generated 3 Clusters. Cluster I consisted of *S. lutetiensis*/*S. infantarius*, Cluster II consisted of *S. gallolyticus* /*S. pasteurianus*/*S. macedonicus*, and Cluster III consited of *S. equinus*. On the basis of 16S rRNA sequence, the isolate RB4 was grouped with *S. lutetiensis* and *S. infantarius*, and RB4 shared the highest homology with *S. lutetiensis* strain 2708 (Accession no: MT611721). Therefore, we assumed that the isolate RB4 was *S. lutetiensis*. **Chen *et al.* (2021)** isolated *S. lutetiensis* from clinical mastitis of dairy cows and concluded that *S. lutetiensis* had a good adaptive ability in bovine mammary cells or tissue. **De Sousa *et al.* (2021)** isolated Streptococci from rumen fluid of Holstein cows and Nellore heifers, and 30% of the isolates belonged to *S. lutetiensis*/*S. infantarius* group. *S. lutetiensis* was also isolated from the rumen fluid of Korean goats **(Park *et al.*, 2021)**.

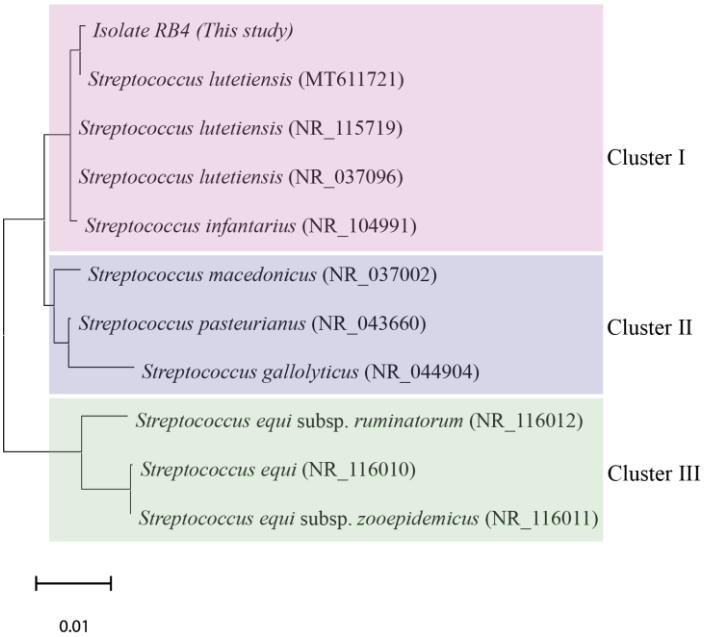

Figure 1 The tree is figured to scale using branch lengths with the units as in the evolutionary distance calculation for the phylogenetic tree formation. This analysis engaged 11 nucleotide sequences. All indefinite positions were subtracted for each sequence (pairwise deletion option). Totally, 1547 positions were left in the final dataset. Accession numbers of sequences were given in parenthesis

Cloning and expression of *amySL*

The length of the DNA fragment amplified with the primers AmySbF - AmySbR was 1640 bp. The DNA fragment was inserted into the pGEM®-T Easy Vector. The constructed vector was named as pGEMTA1 and transformed into *E. coli* DH5α. *E. coli* DH5α did not produce amylase normally, therefore an amylase positive recombinant colony was purified and named as *E. coli* A1 after the transformation. pGEMTA1 was isolated from the recombinant *E. coli* strains and the presence of the DNA fragment in the recombinant plasmid (pGEMTA1) was confirmed by PCR amplification using the AmySbF - AmySbR primer pair. The isolated amylase gene was named as *amySL*. The complete nucleotide sequence of *amySL*, obtained from pGEMTA1, was determined in both strands. *amySL* contained a complete ORF with a length of 729 bp, and encoding polypeptide of 298 amino acids with predicted molecular mass (MW) and isolelectric point (pI) of 33.456 Da and 4.86, respectively. Theoretically, pI value below 7 indicates the acidic nature of the proteins **(Dutta *et al.*, 2018)**. The activity of amylase expressed by AmySL on the E. coli A1 plasmid pGEMTA1 was determined by zymogram analysis The enzyme produced after 12 hours of incubation of *E. coli* A1 strain in

LB medium was loaded onto SDS-PAGE gel. The extract obtained from *E. coli* DH5α strain was also loaded on the same gel as a control. In the zymogram analysis, an activity band at ~30 kDa was observed in *E. coli* A1 strain, while no amylase activity band was observed in *E. coli* DH5α strain. The difference of about ~3 kDa between the MW estimated *in silico* and the MW in the zymogram analysis was thought to be due to the SDS-PAGE conditions (Figure 2). The MW and pI of the amylase enzyme in the genome of *S. equinus* isolated from bovine rumen are 81.48 and 5.06, respectively. This enzyme also contains a starch binding domain (CBM26) **(Azevedo *et al.*, 2015)**. Similarly, the amylase of *S. gallolyticus* UCN34 had a CBM26, and MW and pI values of this enzyme were 81.43 and 4.66, respectively **(Rusniok *et al.*, 2010)**. The MWs of the extracellular and intracellular amylases of *S. bovis* JB1 were calculated as 70 kDa and 56 kDa, respectively **(*Whitehead and Cotta,* 1995).** AmySL of *S. lutetiensis* RB4 does not contain a starch binding domain, and the MW of this enzyme was considerably smaller than the amylases of the previously studied ruminal Streptococci. The optimal pH value was found at 6.5 and there was substantial activity between pH values 6.5 and 8.0 (>90%). A sharp decrease in pH stability above pH 7.5 was observed for AmySL. AmySL activity increased with the increment of temperature from 10 to 40ºC. Maximum activity was obtained at 40ºC and significant decrease was detected from this point to 60ºC. Similar pH and temperature values had been found for the amylase of *S. bovis* 148 and JB1. TLC was used for end product analysis of the amylase with its substrate. AmySL hydrolysis pattern was revealed that the main products were maltose maltotriose, maltotetrose and glucose.On the other hand, glucose was only apparent when the extended hydrolysis was performed using AmySL. The amylase enzyme of *S. bovis* JB1 has been reported to hydrolyze starch to maltooligosaccharides. It was found that the extracellular AmyI enzyme of *S. bovis* 148 hydrolyzes starch into glucose and maltose, while AmyII enzyme hydrolyzes most of the starch to maltotriose and a small portion to maltose **(Satoh *et al.*, 1997).**

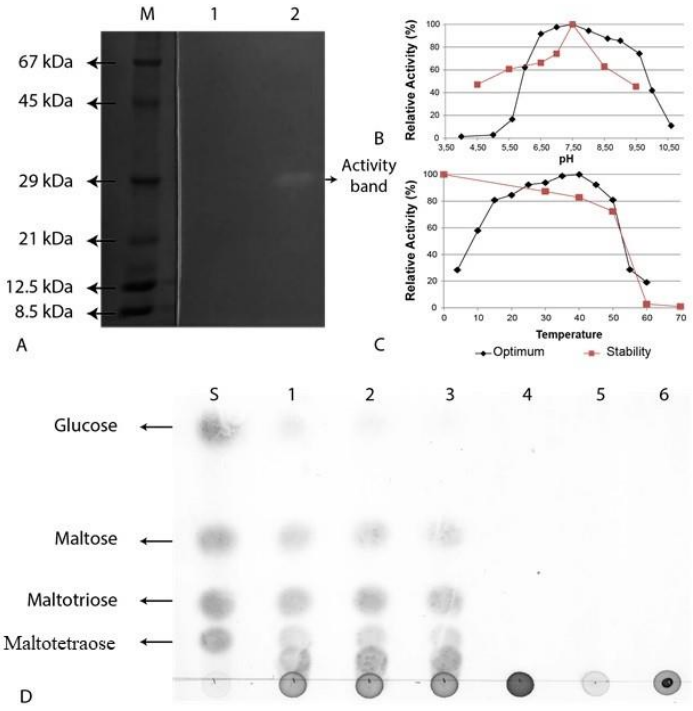

Figure 2 (A) *amySL* expression analysis with 12.5% SDS-PAGE containing 0.5% starch. Lane M: Protein molecularMW markers,, Lane 1: cell lysate of *E. coli* DH5α, Lane 2 : Cell lysate of *E. coli* A1. (B) Optimum pH and pH stability AmySL. (C) Optimum temperature and thermostability of AmySL. (D) TLC of the end-products of soluble starch hydrolyzed by AmySL cloned in *E. coli* A1 at 40ºC and pH 6.5. S: 1 mg/mL standard (Merck, Germany), Lane 1: 60 min incubation, Lane 2: 30 min incubation, Lane 3: 15 min incubation, Lane 4: 0 min incubation, Lane 5: Substrat control (1mg/mL), Lane 6: Enzyme control

In silico analysis of AmySL

Conserved sequence regions of AmySL were analyzed using InterPro and NCBI's Conserved Domain Search tools. AmySL contain the catalytic domain of the GH13 family. The GC content for the ORF of *amySL* was found to be 37.9%. The instability index was 22.69 and this value classified the protein, AmySL, as stable. Aliphatic index and hydropathicity average values were 71.04 and -0.488, respectively. The proteins which have a high aliphatic index tended to be thermostable **(Ikai, 1980)**. The lower range of GRAVY signifies the possibility of better interaction of AmySL with water **(Gupta *et al.*, 2012)**. The major amino acid residues found in AmySL were Asp (8.4%), Gly (7.4%), Leu and Phe (7.0%), Tyr and Ala (6.7%) (Table 1). Asp frequency has been tended to lessen while optimal growth temperature of organisms increases, since it was noticed to be variable at high temperatures **(Kumar *et al.*, 2014)**. Because the isolated *S. lutetiensis* RB4 is a rumen microorganism, its optimum growth temperature is 39-40ºC. This indicated the optimum temperature of AmySL was not so high. On the other hand, a high proportion of glycine and low a percentage of proline and arginine in AmySL increases the possibility of the flexible structure of this enzyme. While lower percentage of arginine and proline in an enzyme contributesto enhancing structural flexibility **(*Latip et al.*, 2019)**, the higher percentage of glycine helps to function as a flexible link in proteins **(Hall, 1998)**. The predicted scale solubility of AmySL was found to be 0.566 and this value was above the threshold level of 0.45 which shows the solubility of AmySL. There is no certainty for the solubility of proteins intended to be produced recombinantly, therefore, solubility estimation has a significant impact on recombinant protein production so that extra costs will be avoided by eliminating insoluble proteins from trials **(Ghomi *et al.*, 2020)**.

Table 1 Amino acid composition of amylase from *S. lutetiensis* RB4

Amino acid	Number	% of amino acid
Asp (D)	25	8.4%
Gly (G)	22	7.4%
Leu (L)	21	7.0%
Phe (F)	21	7.0%
Ala (A)	20	6.7%
Tyr (Y)	20	6.7%
Asn (N)	19	6.4%
Glu (E)	19	6.4%
Lys (K)	18	6.0%
Thr (T)	18	6.0%
Ile (I)	17	5.7%
Val (V)	15	5.0%
Gln (Q)	12	4.0%
Ser (S)	11	3.7%
Arg (R)	9	3.0%
His (H)	9	3.0%
Pro (P)	8	2.7%
Trp (W)	6	2.0%
Met (M)	5	1.7%
Cys (C)	3	1.0%

Random coils (37.25%) and α-helix (34.23%) were dominated the secondary structure of AmySL followed by extended strand (24.50%), and less amount of β-turn (4.03%). More helix regions give the proteins an advantage at high temperature, while the sheet regions are found low in thermophilic protein since they are thermolabile (**Kumar *et al.*, 2014**). AmySL was homology modelled with *Geobacillus stearothermophilus* α-amylase (PDB: 4UZU) and interpreted through the structural assessment server of the Swiss-Model workspace. The sequence identity and coverage between AmySL and *G. stearothermophilus* α-amylase were 45.92% and 99%, respectively. The oligostate of the AmySL was monomer, and GMQE and QMEAN values of AmySL were found to be 0.83 and 0.79, respectively. GMQE score is stated as a value between 0 and 1, and higher numbers define higher reliability **(Biasini *et al.*, 2014)**. Additionally, QMEAN scores of -4.0 or below are a sign of models with inadequate quality **(Benkert *et al.*, 2011)**. Analysis of the Ramachandran plot displayed that 93.17% of residues of AmySL resided in the ideal region. For a good quality model, it is recommended that the residue in the favored region should be above 90% **(Pramanik *et al.*, 2017)**. The protein model of AmySL had overall quality factors of 92.63% according to ERRAT. The Verify 3D of AmySL show that 91.86% of residues have an average 3D-1D score ≥ 0.2. PROCHECK analysis proposed that 97.7% of the total residues of AmySL enzyme are found in favored and allowed regions, which shows that stereochemical factors of the built model are reasonably good. The overall results from ERRAT, Verify 3D and PROCHECK web tools have validated the outstanding 3D protein model quality of AmySL from *S. lutetiensis* RB4. Seven conserved sequence regions (CSR) have been proposed for the α-amylase family **(Janeček, 2002)**. CSR II, CSR III, and CSR IV in AmySL of *S. lutetiensis* RB4 were shown in Figure 3. CSR II and IV contain the catalytic residue aspartate (D), and CSR III contains the catalytic residue glutamate (E).

Phylogenetics analysis of AmySL

The evolutionary relationships of AmySL and other rumen microbial amylase enzymes were resolved with the Maximum Likelihood method and JTT matrix-based model **(Jones *et al.*, 1992)**, and a phylogenetic tree was produced. Since AmySL belongs to the GH13 family, other amylase enzymes were selected from the GH13 family. Phylogenetic analysis was accomplished on the basis of amino acid sequences of the enzymes (Figure 4). Since sequences of amylase enzymes are variable in both length and amino acid sequence composition, analysis was performed on the alignment of only the GH13 catalytic domains of these enzymes.

For this, the conserved catalytic domains were determined using the InterPro web server. The amino acid sequences of a total of 30 enzymes were used for alignment. Cluster I consisted of two groups.

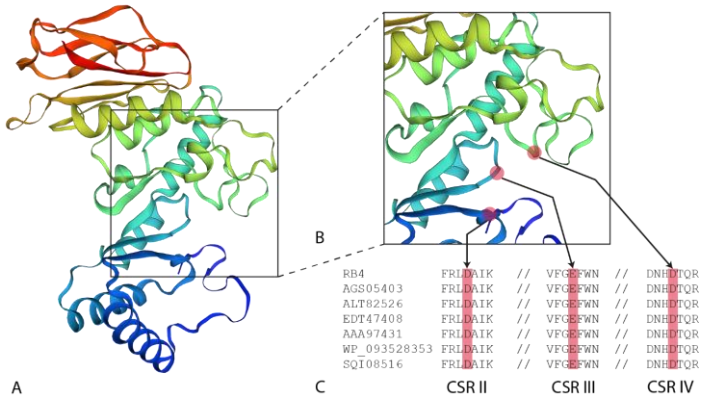

Figure 3 (A) The 3D structure of AmtSL. The structure was viewed using Swiss-Model. (B) Detailed image of the region where the catalytic residues are found. Red circles indicate the location of the catalytic residues. (C) The multiple alignment of *S. lutetiensis* RB4, *S. lutetiensis* 033 (AGS 05403), *S. infantarius* (ALT82526), *S. infantarius* subsp. *infantarius* (EDT47408), *S. equinus* (AAA97431), *S. macedonicus* (WP_093528353), *S. pasteurianus* (SQI08516) amylases. Catalytic residues aspartates (D) and glutamate (E) were indicated with red columns in the CSR II, III and IV

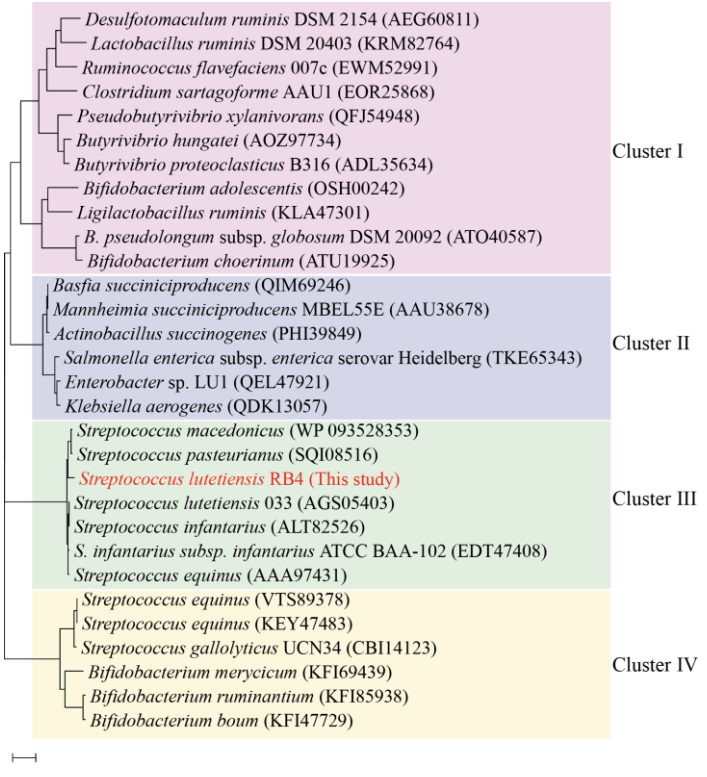

Figure 4 The tree with the highest log likelihood (-16780.43) is indicated. Initial tree(s) for the heuristic search were realised automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 930 positions in the final dataset

The first group included only bacteria belonging to the phylum Firmicutes. In the second group, there were amylases belonging to *Lactabacillus* and *Bifidobacterium*. Although *L. ruminis* belongs to Firmicutes, it was grouped together with *Bifidobacterium*, which belongs to Actinobacteria. Additionally, it was seen that amylases belonging to *Bifidobacterium* were also included in Cluster IV as a second group. Similarly, amylases belonging to *Streptococcus* were also included in two separate groups as Cluster III and IV. Cluster III contained only *Streptococcus* amylases. *S. lutetiensis* RB4 isolated in this study grouped together with *S. macedanicus* and *S. pasterurianus* in Cluster III. Cluster II contains conserved sequence regions belonging to the malS superfamily and consisted only of bacteria belonging to the Proteobacteria phylum. *malS* refers to the gene encoding the periplasmic alpha-amylase of *Escherichia coli* **(Schneider *et al.*, 1992)**. Proteobacteria are the most diverse bacterial phylum (**Moon *et al.*, 2018**) which are predominantly found in the rumen microbiota of cattle together with Firmicutes, Bacteroidetes and Actinobacteria **(Auffret *et al.*, 2017)**. Enzymes belonging to the GH13 family have variety of substrate specificities and catalytic activities, therefore, GH13 has been divided into 43 subfamilies **(Kumar, 2011)**. In the phylogenetic analysis here, amylases are grouped according to their catalytic activities. Amylases in Cluster III containing *Streptococcus* bacteria contain the cytoplasmic alpha-amylase **(Raha *et al.*, 1992)** catalytic domain. Catalytic activities of the Cluster IV group containing *Streptococcus* amylases are included under AmyAc_Bac1_AmyA group.

CONCLUSION

High-concentrate feeds are an important diet for productive ruminant animals and therefore it is important to examine the starch degrading microorganisms in the rumen. The *S.bovis / S.equinus* complex plays a significant role in the hydrolysis of starch in the rumen. This research point out an intention to identify some features of the amylase gene isolated from a bacterium associated with the *S.bovis / S.equinus* complex. Since, the variations in the α-amylase enzyme sequence are pretty much in contrast to the other enzymes of the GH13 family members, *amySL* cloned in this study will add to our understanding of the enzymes involved in the breakdown of rumen starch. Further studies are needed to reveal a broad repertoire of genes encoding amylolytic enzymes of the *S. bovis / S.equinus* complex for better understanding the starch hydrolysis in the rumen and developing strategies against ruminal acidosis.

Acknowledgments: This project was supported by Kahramanmaras Sutcu Imam University BAP (Project number: 2014/3-37D).

REFERENCES

Amaro, F. X., Kim, D., Agarussi, M. C. N., Silva, V. P., Fernandes, T., Arriola, K. G., …& Vyas, D. (2020).Effects of exogenous α-amylases, glucoamylases, and proteases on ruminal in vitro dry matter and starch digestibility, gas production, and volatile fatty acids of mature dent corn grain. *Translational Animal Science*, *5*(1). http://dx.doi.org/10.1093/tas/txaa222

Auffret, M. D., Dewhurst, R. J., Duthie, C-A., Rooke, J. A., John Wallace, R., Freeman, T. C, … & Roehe, R. (2017). The rumen microbiome as a reservoir of antimicrobial resistance and pathogenicity genes is directly affected by diet in beef cattle. *Microbiome*, *5*(1),159-159. http://dx.doi:org/10.1186/s40168-017-0378-z

Azevedo, A. C, Bento, C. B. P., Ruiz, J. C., Queiroz, M. V., Mantovani. H. C. (2015). Draft Genome Sequence of Streptococcus equinus (Streptococcus bovis) HC5, a Lantibiotic Producer from the Bovine Rumen. *Genome Announcement*, *3*(2), e00085-15. http://dx.doi:org/10.1128/genomeA.00085-15

Benkert, P., Biasini, M., Schewede, T. (2011). Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics*, *27*(3), 343-350. https://doi.org/10.1093/bioinformatics/btq662

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., … & Bordoli, L. (2014). SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Research*, *42*(W1), W252-W258. https://doi.org/10.1093/nar/gku340

Chen, P., Qiu, Y., Liu, G., Li, X., Cheng, J., Liu, K., … & Gao, J. (2021). Characterization of *Streptococcus lutetiensis* isolated from clinical mastitis of dairy cows. *Journal of Dairy Science*, *104*(1), 702-714. http://dx.doi:org/10.3168/jds.2020-18347

Colovos, C.,Yeates, T. O. (1993). Verification of protein structures: patterns of nonbonded atomic interactions. *Protein Science*, 2(9), 1511-1519. https://doi.org/10.1002/pro.5560020916

Comlekcioglu, U., Akyol, I., Ozkose, E., Kar, B., Ekinci, M. S. (2008). Carboxymethylcellulase production by the anaerobic rumen fungusNeocallimastix sp. GMLF7. *Annals of Microbiology*, *58*(1), 115-119. http://dx.doi:org/10.1007/BF03179454

De Sousa, B. L., Azevedo, A. C., Oliveira, I. M. F., Bento, C. B. P., Santana, M. F., Bazzolli, D. M. S., Mantovani, H. C. (2021). PCR screening reveals abundance of bovicin-like bacteriocins among ruminal *Streptococcus* spp. isolated from beef and dairy cattle. *Journal of Applied Microbiology*, *131*(4), 1695-1709. http://dx.doi:org/10.1111/jam.15069

Dekker, J. P., Lau, A. F, Kraft, C. S. (2016). An Update on the *Streptococcus bovis* Group: Classification, Identification, and Disease Associations. *Journal of Clinical Microbiology*, *54*(7), 1694-1699. http://dx.doi:org/10.1128/JCM.02977-15

Dutta, B., Banarjee, A., Chakraborty, P., Bandopadhyay, R. (2018). In silico studies on bacterial xylanase enzyme: Structural and functional insight. *Journal of Genetic Engineering and Biotechnology*, *16*(2), 749-756. https://doi.org/10.1016/j.jgeb.2018.05.003

Ghomi, F. A., Kittila, T., Welner, D. H. (2020). A benchmark of protein solubility prediction methods on UDP-dependent glycosyltransferases. bioRxiv:2020.02.28.962894. http://dx.doi:org/10.1101/2020.02.28.962894

Gupta, S. K, Rai, A. K., Kanwar, S. S., Sharma, T.R. (2012). Comparative Analysis of Zinc Finger Proteins Involved in Plant Disease Resistance. *Plos One*, *7*(8), e42578. http://dx.doi:org/10.1371/journal.pone.0042578
Hall, J. C. (1998). Glycine. *Journal of Parenteral and Enteral Nutrition*, *22*(6), 393-398. http://dx.doi:org/10.1177/0148607198022006393
Herrera, P., Kwon, Y. M, Ricke, S. C. (2009). Ecology and pathogenicity of gastrointestinal *Streptococcus bovis*. *Anaerobe*. 15(1), 44-54. http://dx.doi:org/10.1016/j.anaerobe.2008.11.003
Hudson, J. A, Cai, Y., Corner, R. J, Morvan, B., Joblin, K. N. (2000). Identification and enumeration of oleic acid and linoleic acid hydrating bacteria in the rumen of sheep and cows. *Journal of Applied Microbiology*, *88*(2), 286-292. http://dx.doi:org/10.1046/j.1365-2672.2000.00968.x
Ikai, A. (1980). Thermostability and aliphatic index of globular proteins. *The Journal of Biochemistry*, *88*(6),1895-1898. https://doi.org/10.1093/oxfordjournals.jbchem.a133168
Janecek, Š. (2002). How many conserved sequence regions are there in the α-amylase family. *Biologia*, *57* (Suppl 11), 29-41.
Jones, D. T., Taylor, W. R., Thorton, J. M. (1992) The rapid generation of mutation data matrices from protein sequences. *Bioinformatic*, *8*(3), 275-282. http://dx.doi:org/10.1093/bioinformatics/8.3.275
Katoh, K., Rozewicki, J., Yamada, K. D. (2017). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics*, *20*(4), 1160-1166. http://dx.doi:org/10.1093/bib/bbx108
Kumar, V. (2011). Identification of the sequence motif of glycoside hydrolase 13 family members. *Bioinformation*, *6*(2):61-63 http://dx.doi:org/10.6026/97320630006061
Kumar, V., Sharma, N., Bhalla, T. C. (2014). In Silico Analysis of-Galactosidases Primary and Secondary Structure in relation to Temperature Adaptation. *Journal of Amino Acids*, 475839. http://dx.doi:org/10.1155/2014/475839
Laskowski, R. A., MacArthur, M. W., Thornton, J. M., Brock, C. P., Hahn, T., Wondratschek, H., … & Rossmann M. G. (2012). *PROCHECK: validation of protein-structure coordinates* (Volume F). https://doi.org/10.1107/97809553602060000882
Latip, A., Asyraf, M., Abdul Hamid, A. A., Nordin, N. F. H. (2019). Microbial hydrolytic enzymes: in silico studies between polar and tropical regions. Polar Science, 20, 9-18. https://doi.org/10.1016/j.polar.2019.04.003
Lee, M., Jeongs, S., Seo, J., Seo, S. (2019). Changes in the ruminal fermentation and bacterial community structure by a sudden change to a high-concentrate diet in Korean domestic ruminants. *Asian-Australasian Journal of Animal Sciences*, *32*(1), 92-102. http://dx.doi:org/10.5713/ajas.18.0262
Liu, J-R., Yu, B., Zhao, X., Cheng, K-J. (2007). Coexpression of rumen microbial β-glucanase and xylanase genes in *Lactobacillus reuteri*. *Applied Microbiology and Biotechnol*ogy, *77*(1), 117-124. http://dx.doi:org/10.5713/ajas.18.0262
Luthy, R., Bowie, J. U., Eisenberg, D. (1992). Assessment of protein models with three-dimensional profiles. *Nature*, *356*(6364), 83-85. http://dx.doi:org/10.1038/356083a0
Matthews, C., Crispie, F., Lewis, E., Reid, M., O'toole, P. W., Cotter, P. D. (2019). The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut Microbes*, *10*(2), 115-132. http://dx.doi:org/10.1080/19490976.2018.1505176
Miller G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, *31*(3):426-428. http://dx.doi:org/10.1021/ac60147a030
Moon, C. D., Young, W., Maclean, P. H., Cookson, A. L., Bermngham, E. N. (2018). Metagenomic insights into the roles of Proteobacteria in the gastrointestinal microbiomes of healthy dogs and cats. *Microbiology Open*, *7*(5), e00677-e00677. http://dx.doi:org/10.1002/mbo3.677
Park, S. Y, Lee, M., Lim, S. R., Kwon, H., Lee, Y. S., Kim, J. H., Seo, S. (2021). Diversity and Antimicrobial Resistance in the *Streptococcus bovis*/*Streptococcus equinus* Complex (SBSEC) Isolated from Korean Domestic Ruminants. Microorganisms, *9*(1), 98. https://doi.org/10.3390/microorganisms9010098
Pompilio, A., Di Bonaventura, G., Gherard, G. (2019). An Overview on *Streptococcus bovis/Streptococcus equinus* Complex Isolates: Identification to the Species/Subspecies Level and Antibiotic Resistance. *International Journal of Molecular Sciences*, *20*(3):480. https://doi.org/10.3390/ijms20030480
Pramanik, K., Ghosh, P. K., Ray, S., Sarkar, A., Mitra, S., Maiti, T. K. (2017). An in silico structural, functional and phylogenetic analysis with three dimensional protein modeling of alkaline phosphatase enzyme of *Pseudomonas aeruginosa*. *Journal of Genetic Engineering and Biotechnology*, *15*(2), 527-537. https://doi.org/10.1016/j.jgeb.2017.05.003
Raha, M., Kawagishi, I., Muller, V., Kihara, M., Macnab, R. M. (1992). *Escherichia coli* produces a cytoplasmic alpha-amylase, AmyA. *Journal of Bacteriology*, *174*(20), 6644-52. http://dx.doi:org/10.1128/jb.174.20.6644-6652.1992
Rasiah, I. A., Rehm B. H. A. (2009). One-Step Production of Immobilized α-Amylase in Recombinant *Escherichia coli*. *Applied and Environmental Microbiology*, *75*(7), 2012-16. http://dx.doi:org/10.1128/AEM.02782-08
Rusniok, C., Couve, E., Da Cunha, V., El Gana, R., Zidane, N., Bouchier, C., Poyart, C., Leclerq, R., Trieu-Cuot, P., Glaser, P. (2010). Genome sequence of *Streptococcus gallolyticus*: insights into its adaptation to the bovine rumen and its ability to cause endocarditis. *Journal of Bacteriology*, 192(8), 2266-76. http://dx.doi:org/10.1128/jb.01659-09
Satoh, E., Uchimura, T., Kudo, T., Komagata, K. (1997). Purification, characterization, and nucleotide sequence of an intracellular maltotriose-producing alpha-amylase from *Streptococcus bovis* 148. *Applied and Environmental Microbiology*, 63(12), 4941-4. http://dx.doi:org/10.1128/aem.63.12.4941-4944.1997
Schneider, E., Freundlieb, S., Tapio, S., Boos, W. (1992). Molecular characterization of the MalT-dependent periplasmic alpha-amylase of *Escherichia coli* encoded by malS. *Journal of Biological Chemistry*, *267*(8), 5148-54. http://dx.doi:org/10.1016/S0021-9258(18)42473-3
Whitehead, T. R., Cotta, M. A. (1995). Identification of intracellular amylase activity in *Streptococcus bovis* and *Streptococcus salivarius*. *Current Microbiology*, *30*(3), 143-148. http://dx.doi:org/10.1007/BF00296199
Yu, A. T., Shapiro, K., Beneri, C. A., Wilks-Gallo, L. S. (2021). *Streptococcus lutetiensis* neonatal meningitis with empyema. *Access Microbiology*, *3*(9), 000264-000264. http://dx.doi:org/10.1099/acmi.0.000264