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Original Article

Effect of *vgb* gene on microbial chondroitin sulfate production in recombinant *Escherichia coli* pETM6-PACF-*vgb* and physicochemical characterization of produced chondroitin sulfate

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Abstract

Chondroitin Sulfate (CS) is an essential component of the extracellular matrix and is a sulfated glycosaminoglycan structurally composed of a polysaccharide chain consisting of *N*-acetyl galactosamine and glucuronic acid. The use of CS of animal origin is common in pharmacological research. The disadvantages of traditional sources and methods used in the production of CS, which is used in various applications in the medicine, veterinary, pharmacy, and cosmetic sectors, have made microbial production a vital alternative. In this study, recombinant *Escherichia coli* (pETM6-PACF-*vgb*) strain, in which *kfoA, kfoC, kfoF* and *vgb* gene regions are co-expressed, and *E. coli* pETM6-PACF strain, which does not contain the *vgb* gene, were used in the *microbial* production of CS. The *vgb* gene is the region responsible for expressing the bacterial protein *Vitreoscilla* hemoglobin (VtHb). This study investigated the effect of the expression of VtHb in *E. coli* on increasing bacterial cell respiration and, therefore, how ATP production would affect cell growth and the acquisition of chondroitin and microbial chondroitin sulfate (MCS) from biomass. The analysis results determined a 23.07% increase in the amount of MCS produced from the *vgb*+ strain. The presence of *vgb* had positively affected culture age and reproductive kinetics. Spectrophotometric measurements, NMR, HPLC, FT-IR, TGA, DTA, and DSC analyses for the reproductive values and physicochemical characterization of the obtained MCS were applied to discuss this production process. For more detailed results on this subject, future research focused on optimization is needed.

Keywords: Microbial biotechnology, Microbial chondroitin, Recombinant bacteria, *Vitreoscilla hemoglobin*, *vgb* gene.

1. Introduction

CS is a homopolymeric Glycosaminoglycan (GAG) containing a single type of repeating disaccharide unit (4GlcAβ1-3GalNAcβ1-: GlcA- glucuronic acid and GalNAc- *N*-acetylgalactosamine) [1,2]. CS is known for its anti-inflammatory, antithrombotic, anticoagulant, and antioxidant effects. Studies have reported that CS helps support bone formation, accelerate bone healing, block angiogenesis and tumor growth, regulate blood lipids, improve atherosclerosis, repair and regenerate the central nervous system, and joint-related pathologies [3]. Current research on CS is mainly directed to functional food (or food supplements), medicine, pharmacy, biomaterial, cosmetics, etc. [4–10]. It is commonly obtained from animal sources. Current extraction and purification procedures of CS are complex and expensive methods that carry disadvantages such as risks of viral contamination, low product yield, serious contamination caused by many organic chemicals and protein residues, and unstable product quality. In recent years, different strategies have been proposed for the biotechnological production of CS or CS-like products to overcome these problems [11].

This study discusses the effect of the *Vitreoscilla* hemoglobin gene (*vgb*) on microbial chondroitin and microbial chondroitin sulfate (MCS) production in the recombinant *Escherichia coli* pETM6-PACF-*vgb* strain. *Vitreoscilla* Hemoglobin (VtHb), the transcription product of the *vgb* gene, is recommended as a usable agent in the field of metabolic engineering in microorganisms, plants, and animals to perform high cell density fermentation under oxygen-limited conditions, increase product synthesis, and improve stress tolerance. It has been shown that VtHb makes the activation of the electron transfer chain of *E. coli* more effective under hypoxic conditions. It has been determined that such an effect increases cell growth and oxygen uptake not only in *E. coli* but also in other bacteria, yeast and

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transgenic plants [12–17]. The impact of VtHb on function and composition in cells has been studied in detail. It has been shown that VtHb causes an increase in the intracellular levels and activity of cytochrome *bo* ubiquinol oxidase, a more effective energy-providing terminal oxidase, an increase in the pH difference across the cytoplasmic membrane, and an increase in ATP production and use. was determined [18]. It has been observed that hemoglobin synthesis increases 5-10 times in environments containing 10% of the atmospheric oxygen amount. At more critical oxygen concentrations, that is, under microaerophilic conditions, the *vgb* gene is stimulated to the maximum extent, and the intracellular concentration of VtHb increases 40-50 times [12,14,19,20]. Under such conditions, VtHb is thought to play an important role in the growth and proliferation of the microorganism by acting as a buffer against environmental oxygen changes [12]. For many microorganisms, oxygen concentration changes are decisive for growth and proliferation. When the oxygen concentration falls below certain limits, significant changes occur in all cells' physiological and metabolic activities, cell growth often stops, and cell disintegration occurs. Under these conditions, VtHb was observed to prolong the culture period of cells and regulate their metabolic activities. In this study, *E. coli* pETM6-PACF-*vgb* recombinant carrying *kfoA, kfoC, kfoF* and *vgb* gene regions, and *E. coli* pETM6-PACF strain lacking the *vgb* gene were used in the microbial production of CS. This study discussed the effect of the *vgb* gene on chondroitin and MCS production and spectrophotometric measurements used to calculate reproductive values. Nuclear magnetic resonance (NMR), High-performance liquid chromatography (HPLC), Fourier-transform infrared spectroscopy (FT-IR), Thermogravimetric analysis (TGA), Differential thermal analysis (DTA), and Differential scanning calorimetry (DSC) analyses were performed to determine the intramolecular bonds of the obtained MCS, the molecular structure, and the intramolecular functional groups.

2. Material and Methods

In this study, the MCS production protocol was applied to the vgb^+ recombinant *E. coli* pETM6-PACF-*vgb* strain and the vgb^- recombinant *E. coli* pETM6-PACF strain [1, 2, 21]. NMR, HPLC, FT-IR, TGA, DSC, and DTA performed the structural and surface analyses of MCS.

2.1. Preparation of bacterial cell culture

The created recombinant strains carrying the *vgb* gene were also used in previous studies [2, 21]. *E. coli* pETM6-PACF-*vgb* and *E. coli* pETM6-PACF strains were cultivated in Luria-Bertani (LB) medium supplemented with ampicillin (80 mg/L). The medium was prepared using 20 g peptone, 20 g NaCl, and 10 g yeast extract for 2 L. The medium was sterilized by autoclave (Nüve OT 90 L, Turkiye) at 121 Cº for 15 minutes. Bacterial strains were inoculated into the medium and incubated in the incubator (Nüve ES 252, Turkiye) for 48 hours.

2.2. Chondroitin extraction and purification protocol

50 ml falcon tubes were used to precipitate the bacterial cell culture, which was incubated for 48 hours in 2 L Erlenmeyer and centrifuged at 9000 rpm for 5 minutes. During the centrifugation process, the supernatant was discarded each time. Finally, in the centrifuge section, the pellets accumulated in the tubes were collected in a single tube. The tube containing the collected pellet was sterilized in an autoclave at 121 °C for 15 minutes. After sterilization, the pellet was cooled and centrifuged. The supernatant obtained as a result of centrifugation was transferred to another tube, and the supernatant was made up to 45 mL with alcohol cooled at +4 °C and stored at -20 °C. Then, the supernatant precipitated with alcohol was centrifuged at 9000 rpm for 5 minutes at +4 °C. After centrifugation, the pellets in the tubes were collected and suspended in a lysis buffer. The lysis buffer was prepared with 6.05 g Tris, 2.38 g MgCl2, and 0.55 g CaCl for 500 ml. DNAase was added to the samples (1mg/L), and the samples were incubated at 37 ℃ for 1 hour. In the next stage, Proteinase K (2.5 mg/ml) was added to the samples and incubated at 56 ℃ for 2 hours. Cold alcohol (+4 ℃) was added to the sample so that the total volume in the tube was 45 ml. The samples were centrifuged at 9000 rpm for 5 minutes, and the supernatant was discarded. The remaining pellet was dissolved by vortexing in the centrifuge tube with 15 ml of distilled water. It was poured into the plastic container with a volume of 15 ml. It was covered with aluminum foil, frozen, and made ready for filtration. After filtration, the residue was lyophilized in a vacuum for NMR analysis.

2.3. Microbial chondroitin sulfation protocol

In this study, microbial chondroitin sulfation was applied with minor changes to the method used in previous studies [2, 21]. The amount of chondroitin produced is 5.67g for *E. coli* pETM6-PACF-*vgb* strain and 4.62g for *E.coli* pETM6-PACF. The temperature of the chondroitin we obtained was set to 0-5℃. 17.01 g and 13.86 g of Sulfur Trioxide Pyridine were added to 5.67 g and 4.62 g of chondroitin, respectively. When the solution reached room temperature, precipitation was carried out by adding 570 ml of NaCl-saturated acetone for 5.67 g and 460 ml of NaCl-saturated acetone for 4.62 g. The saturated acetone remaining on the precipitate was withdrawn with a pipette, and the remaining precipitate was poured into plastic containers and dried with a lyophilizer. From the solid amounts obtained after the drying process, 5.67 g of chondroitin was separately mixed with 280 ml of deionized water and 1.25 N NaOH; Neutralization was achieved with 200 ml deionized water and 0.75 N NaOH for 4.62 g of chondroitin. HCl was added to the samples to bring the pH value to 7. After neutralization was achieved, the samples were heated to 40 Cº. The solution was filtered through a membrane with a permeability conductivity of less than 10 µs (activated membrane). Finally, the solution was lyophilized and dried.

2.4. High-Performance Liquid Chromatography (HPLC) analysis

High-performance Liquid Chromatography (HPLC) is an analytical chemistry method used to separate the components in a mixture and determine their quality and quantity. In the HPLC analysis of the produced MCSs, commercial CS was used as a control.

2.5. Nuclear Magnetic Resonance (NMR) analysis

NMR analyses of the MCSs we produced in our study were performed with Topsin 3.1.7 software (Bruker) on a Bruker Avance III 600 HD spectrometer (Bruker BioSpin, Billerica, MA, USA). Conditions for one-dimensional 1H

NMR spectra are as follows: flicker field of 12.3 kHz, acquisition time of 2.66 s, relaxation delay of 8.00 s, and temperature of 24.8 °C.

2.6. Spectrophotometric analysis

Spectrophotometric analyses of *E. coli* pETM6-PACF-*vgb* strain and *E. coli* pETM6-PACF strain growth levels were performed with Agilent Technologies Cary 60 UV-Vis (USA) spectrophotometer device.

2.7. Physicochemical characterization analyses

FT-IR, TGA, DTA, and DSC analyses were performed to determine the molecular difference between the MCS produced in our study and commercial CS. FT-IR analysis was performed with the Perkin Elmer UATR Two (USA) device. With the FT-IR device, intramolecular bonds, molecular structure, and intramolecular functional groups can be determined. FT-IR spectroscopy (Perkin Elmer Spectrometer) in the range of 400–4000 cm^{-1} was used to reveal the functional groups of the prepared materials.

TGA, DTA, and DSC measurements were performed with Shimadzu TGA 50 (Japan), Shimadzu DTA 50 (Japan), and Shimadzu DSC-60 (Japan) devices, respectively. TGA is generally used to determine the mass loss and/or gains that occur in materials as a function of temperature or time. DSC measures the energy absorbed or released while the sample is heated, cooled, or kept at a constant temperature. This technique displays the heat difference coming or going away from the reference and the sample depending on temperature or time. DTA is a technique in which the temperature difference (ΔT) between the sample and the reference substance is measured as a function of temperature. In contrast, the same temperature program is applied to the sample and reference substance in a controlled manner. DSC and DTA follow the same analysis path. TGA measurements were performed with approximately 10 mg of the sample under an air atmosphere with a heating rate of 10 °C/min from room temperature to 600 °C. DTA measurements were performed with approximately 10 mg of sample under an air atmosphere with a heating rate of 10 °C/min from room temperature to 600 °C. DSC measurements were carried out with approximately 5 mg of the sample under an air atmosphere with a heating rate of 10 °C/min from room temperature to 400 °C.

2.8. Quantitative analyses

The quantitative analyses conducted in this study focused on evaluating the impact of the *vgb* gene on microbial chondroitin sulfate (MCS) production in recombinant *E. coli* strains. The results indicated a significant increase of 23.07% in MCS yield from the *vgb*+ strain compared to the control *vgb*- strain, demonstrating the positive influence of the *vgb* gene on cell respiration and ATP production, which are critical for cellular growth and metabolite synthesis. Spectrophotometric measurements were employed to assess bacterial growth over time, with OD600 values showing consistently higher readings for the *vgb*+ strain across various time points, further supporting the enhanced metabolic activity attributed to the presence of the *vgb* gene. Additionally, physicochemical characterization through techniques such as NMR and HPLC confirmed that the structural properties of MCS produced by the *vgb*+ strain were comparable to commercially sourced chondroitin sulfate, indicating that genetic modifications can effectively optimize microbial production processes for biotechnological applications.

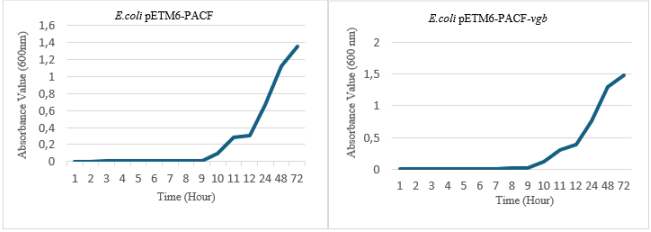
3. Results

3.1 Amount of Produced MCS

In this study, MCS production was carried out using the biomass obtained by inoculating 126 lt liquid media of *E. coli* pETM6-PACF-*vgb* and *E. coli* pETM6-PACF strains separately and the protocol was carried out. The amount of MCS we produced from these groups is 4.96 g per 126 liters for the *E. coli* pETM6-PACF-*vgb* strain and 4.03 g per 126 liters for the *E. coli* pETM6-PACF strain. Between the two strains, higher chondroitin was obtained in the vgb^+ strain.

3.2. Spectrophotometric analysis

Samples from *E. coli* pETM6-PACF-*vgb* and *E. coli* pETM6-PACF strains were prepared and incubated for spectrophotometric measurement. Samples were taken one hour apart between the 1st and 12th hours, and after the 12th hour, Spectrophotometric measurements were made at the 24th, 48th, and 72nd hours. The absorbance values of the samples determined at OD_{600} nm are shown in Fig. 1 and Table 1. The difference between the strains in OD_{600} values and the fact that the vgb^+ strain has higher values than vgb^- shows the effect of the *vgb* gene in anaerobic conditions.



(a) (b)

Fig. 1. OD600 values of E.coli pETM6-PACF (a) and E.coli pETM6-PACF-vgb (b).

Table 1. OD_{600} values of *E.coli* pETM6-PACF and *E.coli* pETM6-PACF-*vgb*.

Time (hour)	*E. coli* pETM6-PACF-*vgb* (OD_{600} nm)	*E. coli* pETM6-PACF (OD_{600} nm)
1	0,0029	0,0014
2	0,0046	0,0023
3	0,0065	0,0039
4	0,0074	0,0037
5	0,0087	0,0045
6	0,0101	0,0054
7	0,0115	0,0067
8	0,0127	0,0074
9	0,0139	0,0085
10	0,1175	0,0897
11	0,3013	0,2872
12	0,3946	0,3013
24	0,7561	0,6665
48	1,2917	1,1226
72	1,4809	1,3544

3.3. NMR analysis

NMR results of MCS produced from pETM6-PACF-*vgb* (Fig. 2A) and *E.coli* pETM6-PACF (Fig. 2B) and Control Commercial CS (Fig. 2C) were demonstrated in Fig. 2. MCS produced for NMR analysis was treated with Chondroitinase ABC enzyme and the disaccharide units in its structure were released. These disaccharide units gave two side-by-side spectra as a result of NMR. These spectra appeared as *N*-acetyl-*β*-galactosamine-6-sulfate, which we sulfated and unsulfated *β*-glucuronic acid units. The 4-S and 6-S units released as a result of enzyme degradation of commercial CS are shown in Fig. 2(A). It has been reported that the NMR spectra of CS-produced microbially by biotechnological methods match the NMR spectra of control commercial CS and CS obtained from other sources in the literature [2, 21]. Commercial CS contains 40% sulfate groups at the C-4 and 60% at the C-6 positions. This study created a sulfate pattern at the 6-position, identical to commercial CS. Additionally, the molecular weight of commercial control CS is stated to be approximately 18,000-20,000 D. In MCS, as a result of NMR analysis, the molecular weight was determined to be in the range of 300-3000 D.

3.4. HPLC analysis

The HPLC analysis results of the MCS produced from *E. coli* pETM6-PACF-*vgb* and *E. coli* pETM6-PACF strains are shown in Fig. 3. The HPLC analysis result of the control commercial CS is shown in Figure 3(C). HPLC results support NMR analysis results.

3.5. FT-IR

In the tests for determining the intramolecular bonds of MCS and commercial CS produced from recombinant strains, determining the molecular structure, and determining intramolecular functional groups, a wide band peak was observed in the commercial CS structure, especially in the 3000-3600 cm^{-1} range, as seen in Fig. 4. This peak arises from the H bond made by the free hydroxyl units of OH groups. Additionally, stretching vibrations belonging to aliphatic CH groups were observed in the 2850-2950 cm^{-1} range. A wide C=O peak at 1600 cm^{-1} was detected due to carboxyl groups. An N-H stress peak is observed at 1538 cm^{-1}. 1360-1398 cm^{-1} CO stretching vibration and COO free stretching vibration were observed. C etheric stretching vibration was detected as a strong band peak, especially between 1120 and 1000 cm^{-1}. The amide peak at 538 cm^{-1} and the CO-NH peaks at approximately 990 cm^{-1} confirm the chondroitin sulfate structure.

Especially in sulfate groups, a double-bond oxygen peak is seen as a source around 1200 cm^{-1}. When these peaks are taken as a reference, the IR spectrum of the produced CS structure confirms that the desired structure has been obtained. Especially the observation of the S=O peak at 1220 cm^{-1} clearly shows that the structure is sulfated. The surface OH number is low only because the molecular mass is negligible. Accordingly, the vibration of the H peaks between 3000-3600 cm^{-1} is low. All other peaks are similar to each other, which confirms the structure. This low molecular mass is expected to be in line with the lit-

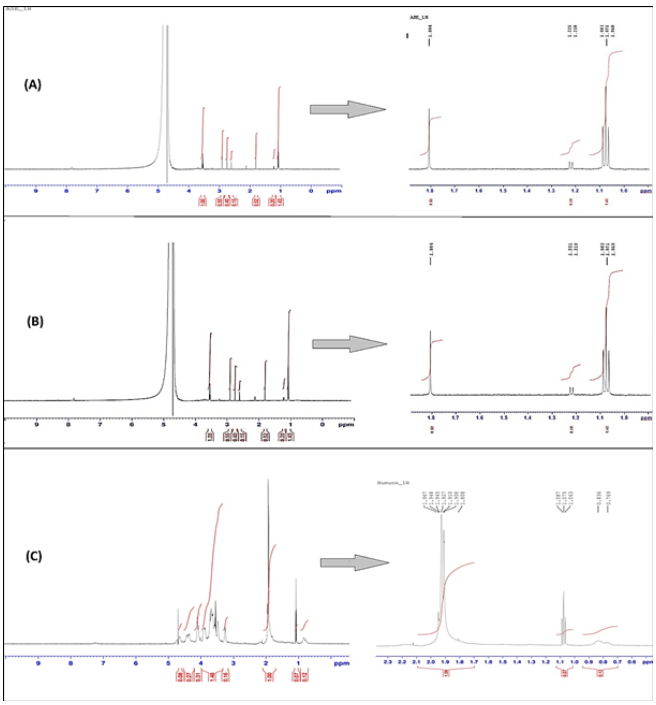


Fig. 2. NMR results of MCS produced from pETM6-PACF-*vgb* (A) and *E.coli* pETM6-PACF (B), Control Commercial CS.

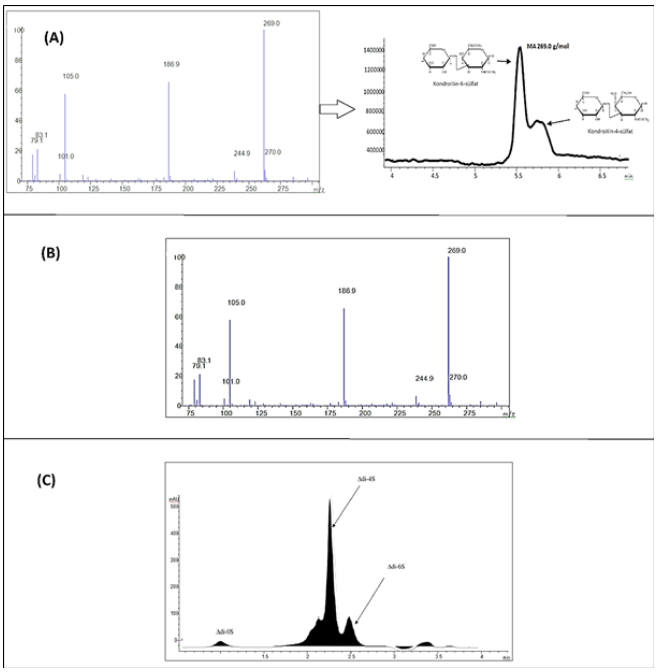


Fig. 3. HPLC results of MCS obtained from *E.coli* pETM6-PACF-*vgb* (A), *E.coli* pETM6-PACF (B), and the control commercial CS sourced from cattle trachea (B).

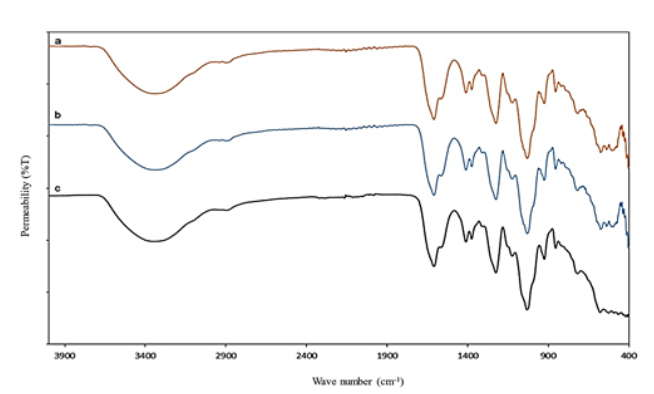


Fig. 4. a) FT-IR analysis results of CS from *E.coli* pETM6-PACF b) MCS from *E.coli* pETM6-PACF-*vgb* c) Commercial CS.

erature. Since the commercial CS structure has a high molecular mass, the peaks are more distinct and intense. It also took the appearance of a band around 400 and 538 cm^{-1}. This prominent peak structure directly indicates the change in molecular mass. In addition, there are significant changes in thermal properties due to the increase in molecular mass in the studies. Therefore, the thermal properties of two different MCS and commercial CS structures were determined using TGA, DTA, and DSC.

3.6. TGA, DTA and DSC

According to the TGA thermogram, three main mass losses were observed in the MCS and Commercial CS samples that we produced from *E. coli* pETM6-PACF-*vgb* strain and *E. coli* pETM6-PACF strain (Fig. 5 (A)). The first mass loss occurs between approximately 60-100 °C and is caused by the removal of structural moisture. As the amount of surface hydroxyls increases, the moisture-binding ability of the relevant structure increases. Therefore, since the commercial CS molecular mass is large, the number of surface hydroxyl groups is high, increasing the peak intensity to approximately 18%. The second mass loss is around 250 and 300 °C. This loss is due to the degradation of side groups in the CS structure. The final mass loss is between 400 °C and 550 °C, indicating the structure's carbonization. All TGA figures have a similar structure, and the MCS shows its thermal stability is around 200 °C.

The DTA thermogram in the figure strengthens the interpretation of TGA. Decreases around 100 °C indicate that structural moisture is moving away. Although this peak was smaller in MCSs, a higher peak was observed in commercial CS (Fig. 5 (B)). A 4-step decomposition structure starts around 200 °C, up to 450 °C, and around 450-500 °C. The degradation of the structural groups of CS causes this degradation. For example, the degradation of 4 units is observed: the degradation of *N*-acetylgalactosamine structures, the degradation of sulfate groups, the degradation of the carbon-oxygen carbon bond, and the degradation of glucuronic acid units. The higher intensity of the peaks in commercial CS is due to its higher molecular mass.

The DSC thermogram shows the change of the MCS and commercial CS structures obtained in our study between 30-400°C. When the DSCs of MCS (a,b) and commercial CS (c) structures are examined, the CS structures show low intensity and similar peaks (Fig. 5(C)) Therefore, it is understood that both structures show the same chemical properties.

4. Discussion

Microbial metabolism is modified using metabolic engineering methods to increase the production of natural metabolites or to produce recombinant metabolites [14,22–24]. The advantages of microbial synthesis applications are limiting the use of environmentally harmful chemicals in chemical synthesis, eliminating the risk of interspecies viral contamination in animal extraction, and preserving ecological balance [25]. Physicochemical characterization of the obtained products and their equivalence to animal and synthetic sources should be supported. Our research discusses the effect of *vgb* expression on this cultivation and product process. It is known that the VtHb protein, which has been extensively studied in many studies regarding its structure and function, increases the productivity of specific metabolic pathways that require oxygen through *vgb* gene expression. With this feature, VtHb is a recombinant protein used in various applications [12]. In our study, in the spectrophotometric analysis, we made for the OD600 values of recombinant *E. coli* pETM6-PACF-*vgb* and *E. coli* pETM6-PACF, which were previously created by our team, we found that the OD 600 absorbance values of the *E. coli* pETM6-PACF-*vgb* strain were lower than those of *E. coli* pETM6-PACF. It was found to be higher than the strain. When both strains were compared regarding the CS ratios we obtained, it was determined that the *E. coli* pETM6-PACF-*vgb* strain caused 23.07% more MCS production. The physicochemical characterization of the product was also analyzed in detail and proved to be identical to its animal counterpart. In the MCS production process, where capsular polysaccharide is the primary substrate, the more biomass is obtained, the more MCS is produced [26]. The contribution of the *vgb* gene to high MCS by regulating metabolism in advanced culture periods and prolonging the culture age is quite obvious. Studies in the literature strongly support our results [13]. VtHb ensures the survival of *Vitreoscilla*, an obligate aerobic bacterium, in oxygen-depleted environments such as muddy puddles. This shows the value of VtHb in the fermentation industry, where aerobic microorganisms are

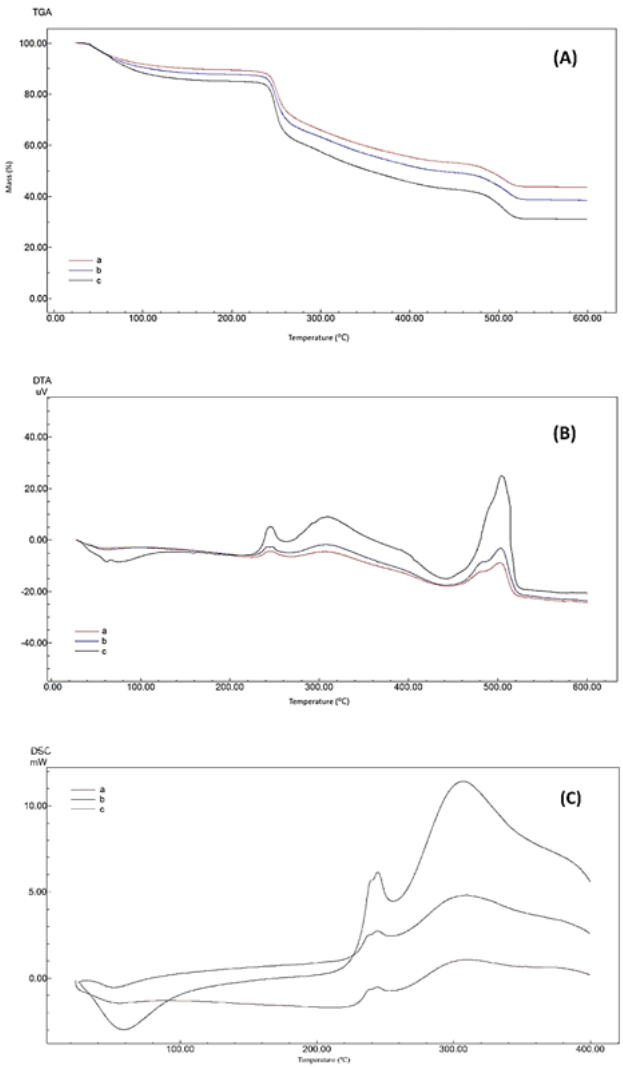


Fig. 5. Results of TGA (A), DTA (B), DSC (C). a) MCS produced from *E.coli* pETM6-PACF b) MCS produced from *E.coli* pETM6-PACF-*vgb* c) Commercial CS.

mostly preferred.

VtHb supports respiration and energy metabolism by promoting oxygen transfer to intracellular terminal oxidases under limited oxygen conditions in fermentation processes with high cell density [27]. It has been stated that the aerobic effects of VtHb in *Saccharomyces cerevisiae* are linked to the electron transport chain [28], and it has been stated that VtHb increases the viability, biomass, and recombinant protein production of *Pichia pastoris* under aerobic conditions [29]. VtHb was developed by *Agrobacterium* sp. to produce Salecan, an exopolysaccharide produced by *Agrobacterium* sp. The *vgb* gene was transformed and inserted into the bacteria and recombinant *Agrobacterium* sp. It was noted that Salecan increased production efficiency by 30% in ZX09 [13]. In another study, vitamin E production was studied, and it was stated that the strain containing VtHb showed approximately two times higher extracellular vitamin E production than the strain without VtHb [30]. These results coincide with our data.

The effect of VtHb on function and composition in cells has been the subject of a series of studies. In these studies, it was determined that VtHb caused an increase in the intracellular levels and activity of cytochrome *bo* ubiquinol oxidase, a more effective energy-providing terminal oxidase, an increase in the pH difference across the cytoplasmic membrane, and an increase in ATP production and use [27]. In addition, the fact that cells containing this protein are more oxidized is related to the fact that VtHb causes significant rearrangements in the carbon metabolism flow schemes of cells. Significant changes in NAD^+/NADH and ADP/ATP ratios are thought to be essential for cell metabolism. In cells containing VtHb, a decrease in the level of NADH and an increase in the amount of ATP are observed. Therefore, significant changes in the NAD^+/NADH and ADP/ATP ratios cause significant changes in how the cell uses carbon compounds [12]. In this context, the increase observed in strains expressing VtHb may be due to such a metabolic flow scheme change. Chondroitin is a structural component of the bacterial cell, so the increase in biomass is significant for the CS process [23,31–35). High biomass means high MCS production. In this study, the effect of *vgb* was clearly observed, and its contribution to the high MCS production process was determined in parallel with the high chondroitin rate. Transferring large DNA fragments and increasing plasmid sizes often slow bacterial metabolism. Relevant gene expression is not affected on a cellular basis, but the culture time is shortened. The *vgb* gene and the resulting VtHb protein offer an ideal solution for this handicap of Recombinant DNA technology**.**

The limitation of this study is that our study is *in vitro*, and there is a risk of contamination occurring *in vitro* studies. There is a margin of error in manual processes during the applied protocol. In this study, the process of obtaining microbial chondroitin and the sulfation process of the obtained chondroitin make the study a two-step study. This process causes a higher need for consumables and an increased risk of error. Therefore, the lengthy and time-production protocol steps may also affect the purity level of the obtained microbial chondroitin sulfate. Further studies are planned to work on the direct biosynthesis of microbial chondroitin sulfate without the sulfation step. Thus, these negative possibilities can be minimized.

The potential applications of this study's findings in pharmaceutical biotechnology research are significant, as they provide the first determination of the effects of the *vgb* gene, which has a rich history in the literature, on chondroitin/chondroitin sulfate production. The physicochemical kinetics of the obtained MCSs were also analyzed in detail, and their identity with commercial CS of bovine origin was revealed. These identities are necessary and meaningful data for the safe use of MCSs in pharmaceutical preparations in the future. Creating a perspective for recombinant GAG production in addition to recombinant proteins in pharmaceutical biotechnology research will be possible with the spread of similar research.

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Conflict of interest

The authors declare no conflict of interest.

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