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Whey-to-Bioethanol Valorisation: Fermentation with Immobilized Yeast Cells

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Abstract

Bioethanol production is gaining prominence as a viable biofuel option, addressing the growing need to reduce fossil fuel dependence. As a viable alternative to produce bioethanol, cheese whey arises as a viable and cost-effective substrate for investigation. This study assessed the organoleptic properties and taxonomic composition of three whey samples: "Merke diary" (technical), "Amiran" (commercial), and "Stella Alpina" (commercial). Highly promising strains from the whey samples, *Kluyveromyces marxianus* A1 and A2, and *Saccharomyces cerevisiae* M1 and M2 were identified by genetic analysis. The immobilization of *Kluyveromyces marxianus* A1 cells with superior adsorption rates on k-carrageenan for 96 hours yielded the highest bioethanol production. Thus, this research signifies the potential of whey as a valuable resource in bioethanol production, aligning with the global shift towards a circular economy.

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1. Introduction

In recent decades, bioethanol has garnered substantial attention within the scientific community. It is increasingly recognized not only as a raw material for biofuel production but also as a promising alternative, primarily due to its outstanding features such as reduced environmental impact, superior octane rating, enhanced biodegradability, and, notably, its capacity to harness renewable resources for its synthesis.[1-5] Currently, global ethanol production exceeds 110 billion L y^{-1}, with approximately 60% utilized in petrol blends, 25% by the chemical industry, and 15% by the food industry. The leading ethanol producers on a global scale are Brazil, the USA, and China. The primary sources for bioethanol production are derived from plant biomass processing waste.[6] Nowadays, yeast cells, predominantly *Saccharomyces cerevisiae*, serve as catalysts to produce bioethanol.[7]

In the dairy industry, milk serves as the primary raw material, but processing generates secondary raw materials and byproducts, including whey, skim milk, buttermilk, rinses, separator sludge, and stripping, which constitute the primary sources of wastewater pollution.[4,6,8,9] Rational utilization of these byproducts is crucial to reduce treatment costs and mitigate the environmental impact.[1,10,11] Approximately half of milk solids end up in whey, resulting in significant nutrient loss (45 kg of fat, 120 kg of protein, 720 kg of milk sugar, *etc*.). Amongst the available options, fermenting whey with lactose-fermenting yeast cultures for ethanol production is the most viable and researched approach.[8,12-14]

The rising demand for ethanol drives the search for cost-effective substrates, with whey, rich in lactose, being a promising option. In the last 30 years, research has focused on using yeasts like *Kluyveromyces fragilis*, *K. marxianus*, and *C. pseudotropicalis* for lactose-based ethanol production, including industrial applications.[15-18] Furthermore, significant attention has been given to *S. cerevisiae* for lactose fermentation, employing strategies like fermenting pre-hydrolyzed lactose solution mixtures. Additionally, lactose-fermenting *S. cerevisiae* strains have been developed using methods such as protoplast fusion and heterologous β-galactosidase expression.[19-21]

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In the past two decades, extensive research has focused on developing immobilized microbial cells as biocatalysts.[22-29] Whey is an economically benign substrate, and bioreactors employing immobilized microbial cells can achieve 3-50 times higher productivity levels. Therefore, employing a bioreactor with immobilized yeast cells enables continuous whey fermentation under optimal conditions, resulting in a high bioethanol yield. Utilizing insoluble carriers for cell and enzyme immobilization facilitates the production of a contamination-free product that can be reused and easily separated from reagents. Numerous literature data reported utilization of various natural microorganisms to immobilize on solid surfaces: *Methylosinus sporium* and *Methylosinus trichosporium* - in methanol production,[30,31] *Rhizopus oryzae* and *Lactobacillus casei* - in lactic acid production,[32-35] S*accharomyces cerevisiae, Mucor circinelloides, Rhizopus oryzae, Fusarium oxysporium,* and *Zymomonas mobilis* - in ethanol production from whey, agricultural, and industrial waste.[36-38]

Thus, the current research examines the organoleptic and taxonomical characteristics of three types of cheese whey (one technical and two commercial) to identify yeast strains suitable for bioethanol production. The subsequent objective was to improve strain performance via cell immobilization on Na alginate beads, polyvinyl alcohol, and kappa-carrageenan.

2. Materials & methods

2.1 Whey origin

The research employed three types of cheese whey as follows: A - LLP “Merke diary” (technical), Oytal village, Zhambyl region; B – LLP “Amiran” (commercial), Almaty; www.amiran.kz; and C – LLP “Stella Alpina” (commercial), Almaty. All the above-mentioned cheese wheys were sampled within the Republic of Kazakhstan.

2.2 Determination of whey organoleptic and physicochemical properties

Sampling of milk whey was performed according to GOST 33957-2016.[39]

Organoleptic indicators were determined through sensory analysis, assessing taste, smell, texture, appearance, and color, following the specifications outlined in GOST R 53438-2009.[40] The evaluation was conducted immediately after sampling and following storage and transportation at a temperature of 2-6 °C for no longer than 8 hours.

To assess appearance and color, a portion of whey was transferred into a clean and dry Petri dish, which was approximately halfway filled. The dish was placed on a white paper sheet and examined under reflected light rays.

Smell and taste properties were evaluated by smelling and tasting the samples. A glass containing a test sample was brought to the nose at a distance of 1-2 cm. The smell was determined by taking two short, deep inhalations. To assess taste, at least 10 cm of the product was consumed, distributing it in the oral cavity to the base of the tongue and holding it for approximately 7 seconds. The sample was then spat out into a spittoon. Swallowing was followed by exhaling through the nose, and a final assessment of the smell and taste was made. The oral cavity was rinsed thoroughly with weakly brewed tea at 35 ± 5 ℃.

The consistency of milk whey was determined by pouring it from a transparent flask (~ 100 mL) into a similar flask while observing the homogeneity of the liquid. The inner walls of the pouring dishes were carefully examined for the presence of protein flakes or other substances.

Titratable acidity was determined by titrating whey, which contains salts, proteins, carbon dioxide, and other compounds, with sodium hydroxide (NaOH) according to GOST 3624-92.[41] The acidity was measured in Turner degrees (°T). To determine titratable acidity, 10 cm^3 of whey containing three drops of a 1% phenolphthalein solution were measured in a 150 cm^3 conical flask. The solution was thoroughly mixed and titrated with 0.1 N NaOH until a stable pink color appeared. This titration process was repeated three times, and the average volume of 0.1 N NaOH was utilised to calculate the acidity using the following equation:

$$x = \frac{n \times 100}{m} \quad (1)$$

where x - acidity; n - volume of 0.1 N NaOH (cm^3); m - mass of whey (g); 100 - a coefficient required for conversion to 100 g of the product.

The active acidity (pH) of whey was assessed using a laboratory pH meter C931P (Consort, Belgium). pH measurements were conducted every 24 hours for 3 days.

The moisture content of whey samples was determined using a MAC 110.X moisture analyzer (Radwag, Poland). The measurements were conducted at a temperature of 110 ℃, with a sample volume of 5 mL. The mass of the dry matter (DM) was determined by repeatedly measuring the sample until it reached a state of complete dryness.

2.3 Determination of whey microbial taxonomic composition

To identify physiological groups, the number of microorganisms was determined using successive sample dilutions on solid nutrient media. The Koch method was employed to determine the colony-forming units (CFU) of microorganisms. This method involves inoculating a specific volume of the microbial suspension onto solid media in Petri dishes and counting the resulting colonies after incubation. After incubation, the colonies were counted, and the number of CFU per gram of the sample was determined.

The qualitative and quantitative analysis of the microbial flora in the natural substrates followed conventional microbiological methods. Mesophilic agar (MPA) was utilized to determine the total count of mesophilic aerobic and facultative anaerobic microorganisms (MAFAMs). Specific nutrient media were used to determine the count of different physiological groups of microorganisms. Sabouraud, MRC (Man, Rogoza, Sharp), and MPA media were employed for

yeast isolation. The cultures were incubated in a thermostat TS-1/20 SPU (Smolensk SDTB PCS, Russian Federation) at a temperature range of 28-30 °C for 2–5 days. Sabouraud medium, consisting of glucose (40.0 g L^{-1}), peptone (10.0 g L^{-1}), agar (18.0-20.0 g L^{-1}), and autoclaved tap water (1 L) served as the nutrient medium for yeast cultivation.

2.4 Pure culture isolation

Pure cultures of microorganisms were obtained through mechanical separation on a solid nutrient medium using the stroke method with loop firing.[42] Microscopic examination was conducted to verify the purity of individual colonies, which were then plated onto culture agar slants for further cultivation (2-5 days).

The determination of morphological, cultural, physiological, and biochemical characteristics of the microorganisms followed established methods.[42] For yeast, the following features were assessed: cell shape, arrangement, and size, colony description on solid nutrient media, and growth patterns in a liquid nutrient medium and on slant agar. Physiological and biochemical properties were evaluated, including yeast thermostability at temperatures of 20, 28, 37, and 45 °C, resistance to different concentrations of NaCl (2, 4, and 6%), and fermentation of various carbohydrates. The identification of yeast cultures was performed according to Bab'eva & Golubev.[43] All experiments for each yeast type were performed in triplicate to verify the effectiveness of the chosen methods.

2.5 Molecular genetic identification

DNA isolation from yeast strains was carried out using the plant/fungi DNA Isolation Kit (Norgen Biotek Corp., Ontario, Canada) following the manufacturer's protocol. The DNA concentration in the samples was determined using the QubitTM dsDNA HS Assay Kit fluorometer (Life Technologies, Oregon, USA), which is specifically designed for high-sensitivity double-stranded DNA. Universal primers targeting the fungal ITS region were used for amplifications: *ITS1* (5'-TCCGTAGGTGAACCTGCGG-3') and (5'-TCCTCCGCTTATTGATATGC-3'). The PCR reaction mixture contained 12.5 µL of Q5® Hot Start High-Fidelity 2X Master Mix, 1.25 µL of forward primer (10 µM), 1.25 µL of reverse primer (10 µM), 1.5 µL of DNA, and 8.5 µL of water, resulting in a total volume of 25 µL.

PCR amplification was performed using an Eppendorf ProS amplifier (Hamburg, Germany) with the following cycling parameters: 94 °C for 30 seconds, 55 °C for 1 min, 72 °C for 40 seconds for 30 cycles, and a final extension step at 72 °C for 10 min. The amplified products were visualized on a 1.2% agarose gel. To purify the PCR products, the CleanSweep™ PCR purification reagent (Applied Biosystems, USA) was utilized.

For the sequencing reaction, the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) was employed, following the manufacturer's instructions. The resulting sequencing fragments were separated on an automatic genetic analyzer 3500 DNA Analyser (Applied Biosystems, USA).

The nucleotide sequences of the ITS region from the yeast DNA were compared to the information available in the Gene Bank database (www.ncbi.nih.gov) using the BLAST program for sequence alignment and identification. Phylogenetic analysis was conducted using the MEGA6 software. Nucleotide sequence alignment was performed using the ClustalW algorithm, and the phylogenetic trees were constructed using the Neighbour-Joining method.

2.6 Cell immobilization

Multiple types of carrier materials were chosen to ensure comparable results. Each selected carrier material was handled based on specific principles and procedures.

Batch fermentation of free cells. A parallel experiment was conducted using free cells to compare the properties of the immobilized cells. The mass of cells used in the immobilized granules was matched in the free-cell experiment. Both experiments were performed under identical conditions, using either glucose or sucrose as the carbon source.

Na-alginate beads. A 3% (w/v) sodium (Na) alginate solution was prepared by dissolving it in 100 mL of autoclaved tap water (1-1.5 Pa for 1.5 h). This solution was then added to a 100 mL suspension of *S. cerevisiae yeast culture* in a beaker and gently shaken. A $CaCl_2$ solution with a final concentration of 2% (w/v) was prepared in a separate beaker. The mixture containing cells and Na-alginate was slowly added dropwise using a 10 mL syringe into the 150 mL $CaCl_2$ solution. The beads were left to solidify in the $CaCl_2$ solution for 1 hour. After solidification, the granules were washed with autoclaved tap water (1-1.5 Pa for 1.5 h) and kept for further use in fermentation experiments. The resulting beads had a diameter of ~ 3-4 mm.

Polyvinyl alcohol (PVA). A daily yeast culture was prepared and cultivated in a thermoshaker at 30 °C and 150 rpm for 24 hours using a Sabouraud liquid medium. To isolate the yeast biomass, the daily culture was centrifuged at 3,500 rpm for 15 min. The supernatant was carefully poured off without stirring, and then 1 mL of autoclaved tap water (1-1.5 Pa for 1.5 h) was added for resuspension.

For carrier preparation, 2.2 g of 11% PVA was dissolved in 20 mL of autoclaved tap water (1-1.5 Pa for 1.5 h) using a water bath at a temperature range of 75-95 °C. The resulting solution yielded 20 mL of carrier. Subsequently, 2 mL of the yeast biomass was added to 20 mL of the PVA carrier and thoroughly mixed. The resulting material was then placed in a refrigerator for 24 hours.

Kappa-carrageenan. To prepare the carrier, 0.6 g of 3% k-carrageenan was dissolved in 20 mL of autoclaved tap water (1-1.5 Pa for 1.5 h) using a water bath at a temperature range of 75-95 °C. The resulting solution yielded 20 mL of k-carrageenan carrier. Then, 2 mL of yeast biomass was added to 20 mL of the k-carrageenan carrier and thoroughly mixed.

The resulting material was then placed in a refrigerator for 24 hours to allow for proper solidification and maturation.

2.7 Analytical methods

Cell sorption. The degree of sorption of cells was determined by measuring the concentration of cells in the solution using an HP/Agilent 8453 UV/Vis spectrophotometer (Agilent Technologies, USA) at a wavelength of 600 nm. A calibration curve was constructed to establish the relationship between the number of cells and the corresponding optical density (OD) values (Fig. S1). The number of immobilized cells was calculated based on the optical density readings of the initial and experimental samples.

The percentage of adsorbed cells was determined by the following formula:

$$R = 100\% \times \frac{D_T \times 100}{D_B} \quad (2)$$

where D_T - OD of the experimental samples; D_B - OD of the initial samples.

The concentrations of ethanol, glucose, and sucrose were measured using high-performance liquid chromatography (HPLC) equipment (1200 series, Agilent Technologies, USA). An Aminex® HPX-87H column from Biorad was used along with a refractive index detector (RID) for detection.

Cell growth was assessed by measuring the optical density at 600 nm using an HP/Agilent 8453 UV/Vis spectrophotometer (Agilent Technologies, USA). The fermentation medium was used as a blank.

2.8 Statistical analysis

The data analysis was performed using RStudio software (version 2022.07.2+576 “Spotted Wakerobin”, RStudio PBC, 2022). Tukey HSD tests were performed for pairwise comparison of means when ANOVA showed a significant effect of the tested factors. Significance was declared at $p < 0.05$.

3. Results & discussion

3.1 Organoleptic and physicochemical characteristics of whey

The sensory evaluation of whey revealed that both A and B whey exhibited a clean, milky taste and smell (Table 1). The texture of these whey samples was homogeneous and opaque, without any presence of precipitates. The color ranged from white to light yellow. On the other hand, C whey exhibited a salty taste and a distinct cheese aroma. It had a uniform texture, appearing as an opaque liquid without any precipitates or flakes. The color of C whey varied between yellowish and pale green. Thus, it can be concluded that the whey products from the selected manufacturers demonstrated good quality.

In the case of nutritional composition, whey samples studied exhibited the following ranges of fats, proteins, and carbohydrates: 0.2-0.4%, 0.5-0.8%, and 3.2-4.2%, respectively (Table 2). According to the fat and protein content consistent with published research whey samples can be considered acid whey.[44-46] The energy value of the whey samples ranged from 18 to 36 Kcal g^{-1}. When comparing technical whey (A) with commercial whey samples (B and C), the increased or equal content of fats and carbohydrates was observed, confirming its feasibility for bioethanol production.

Table 1. Organoleptic characteristics of the studied whey samples.

Parameter	A	B	C
Taste and smell	pure milky taste and smell	pure milky taste without foreign tastes, milky smell	salty taste, cheesy smell
Appearance and texture	homogeneous opaque liquid without precipitates and flakes	homogeneous opaque liquid without precipitates and flakes	homogeneous opaque liquid without precipitation
Color	yellow	beige	yellowish or pale green

Table 2. Physico-chemical properties of the studied whey samples.

Parameter	Unit	A		B		C	
Fats	%	0.40	±	0.20	±	0.40	±
		0.02		0.01		0.02	
Proteins	%	0.50	±	0.80	±	0.70	±
		0.02		0.03		0.03	
Carbohydrates	%	4.20	±	3.20	±	3.90	±
		0.16		0.13		0.15	
Energy	Kcal g^{-1}	28.0	±	17.8	±	36.0	±
		1.20		0.71		1.40	
Moisture	%	92.0	±	92.1	±	93.0	±
		1.80		1.80		1.90	
Dry matter	g	8.00	±	8.00	±	7.00	±
		0.16		0.16		0.14	

Moreover, the moisture content of whey samples fell within the range of 92.0–93.0%, with the DM per 100 g ranging from 7 to 8 g (Table 2).

The titratable acidity of whey tends to rise over time (Fig. 1a). Whey A showed notable titratable acidity levels, ranging from 23 to 75 °T. Whey B exhibited acidity between 65 and 98 °T, while whey C fell within 19 to 68 °T. The titratable acidity across the whey samples spans 19 to 98 °T. Cheese whey typically holds about 20 °T of titratable acidity, while curd and casein whey range from 70 to 75 °T.

The relationship between titratable acidity (measured in Turner degrees) and active acidity (true acidity, pH), which reflects hydrogen concentration, intrigues numerous scientists.[47-49] In whey, the average pH for active acidity lies between 4.0 and 5.0. Notably, acidity is influenced by factors like milk source properties, temperature, and storage duration, leading to decreasing pH values over time.

Additionally, whey is clarified and adjusted for acidity when crafting a nutrient-rich medium, like in bacterial starter culture production. For instance, achieving a pH of 6.0 to 6.5

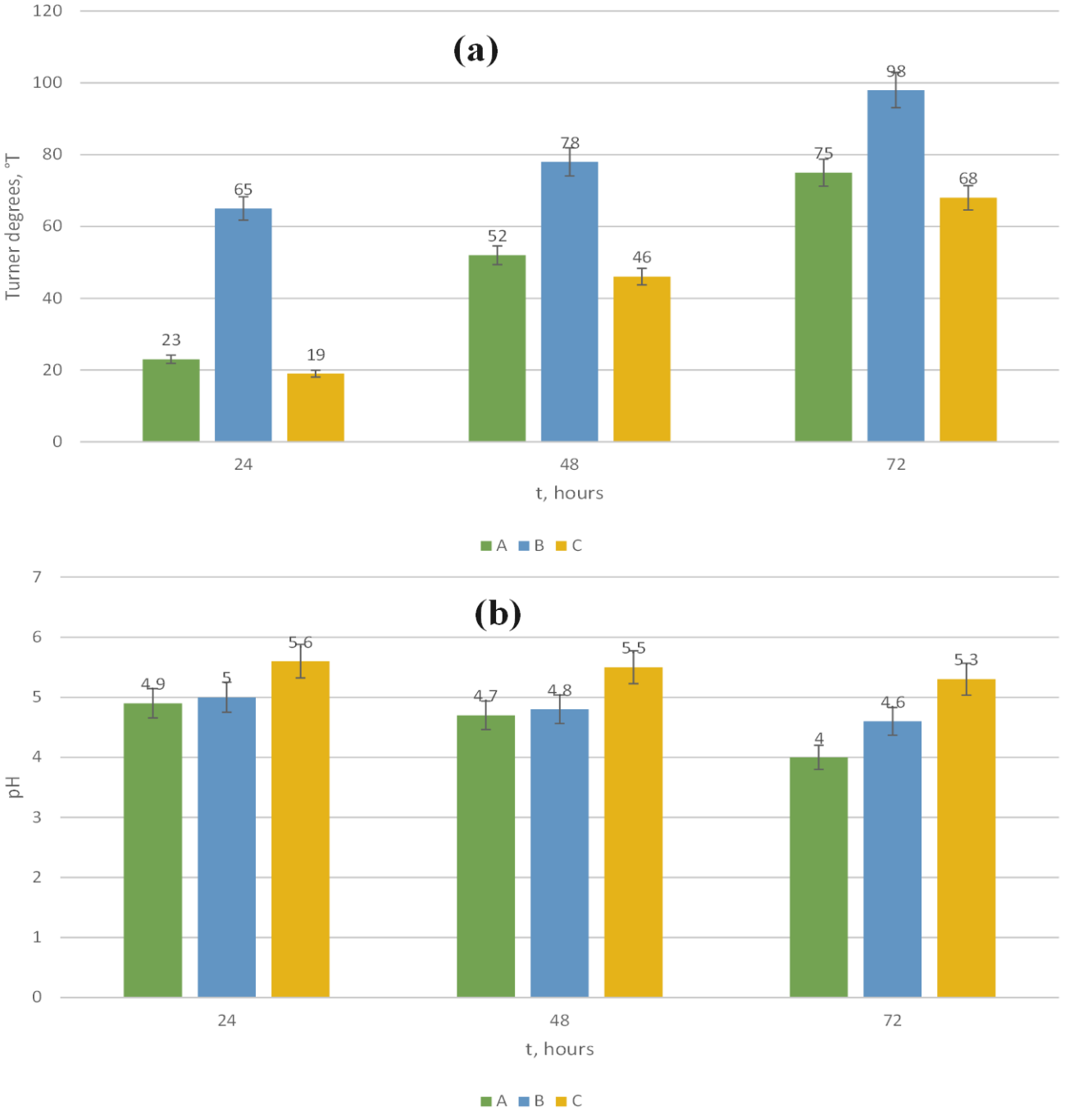


Fig. 1 Titratable and active (pH) acidity dynamics of research whey for 72 hours.

or higher. This results in a notable pH difference between the original whey and the processed form.

The pH of whey B decreased from 5.0 to 4.6. Whey C exhibited an active acidity range of 5.3 to 5.6, and for whey A, the pH spanned from 4.0 to 4.9 (Fig. 1b). In our study, we examined the physicochemical and organoleptic traits of whey and established its suitability for bioethanol production.

3.2 Microbial indicators and taxonomic compositions of research whey

Cheese whey serves as a beneficial nutrient-rich medium for cultivating microorganisms that originate from both the residual heat-resistant and thermophilic microflora found in pasteurized milk, as well as the microflora present in the starter cultures used during production. The composition of cheese whey microflora can vary based on the methods used. Notably, the whey contains both distinctive starter microflora and external elements like heat-resistant granular sticks that persist after pasteurization.

The microflora in fresh cheese whey consists of lactic acid microorganisms, yeasts, and spore-forming rods, and it still retains heat-resistant microflora, encompassing both cocci and rod-shaped forms. The microflora of cheese whey, particularly in the form known as coliform bacteria, include lactose-

fermenting lactic acid microorganisms, heterofermentative lactic acid bacteria, heat-resistant microflora, starter microorganisms, yeasts, and molds. Furthermore, the microflora of cheese whey comprises mesophilic starter culture microflora, manifesting as cocci, diplococci, and rod-shaped forms.[50,51]

Figure 2 illustrates the taxonomic composition of the microbial communities within three distinct whey samples. The Koch method was utilized to discern the microflora in these whey samples and obtain uncontaminated cultures of yeast and lactobacilli.

The lactic acid bacteria (LAB) CFU count on the solid MRS nutrient medium ranged from 2.5 to 7.2 × 10^7 CFU mL^{-1}. On the SDA medium, the count of yeast colonies ranged from 2.8 to 3.9 × 10^7 CFU mL^{-1}. On the universal MPA medium, the count of heterotrophic microorganisms ranged from 1.2 to 2.8 × 10^7 CFU mL^{-1}, notably lower than selective media. It may be attributed to lactic acid microorganism prevalence within the whey microflora.

Whey A contained approximately equal amounts of LAB and heterotrophic microorganisms, while commercial whey samples (B and C) represented similar trends: LAB had the lowest share of the composition and heterotrophic microorganisms – the highest (Fig. 2). In addition, whey B was taken as a control sample to analyze microbial community abundance on phylum and genus levels (Fig. 3). *Firmicutes* and *Proteobacteria* were prevailing phylum in whey B (Fig. 3a), specifically the representatives of *Lactococcus*, *Acetobacter*, and *Lactobacillus* genera (Fig. 3b).

Firmicutes, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* emerge as the key microbial groups in whey, regardless of fermentation at varying temperatures. The same result was obtained by Mazora-Manzano *et al*. (2022, 2020).[52,53] However, incubation temperature distinctly influences overall bacterial diversity during spontaneous whey fermentation. LAB from the *Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Streptococcus* genera predominantly inhabit both fresh and fermented whey. Other non-lactic bacteria, such as *B. subtilis*, *G. thermoleovorans*, *S. aureus*, *S. salivarius*, *A. macleodii*, and *K. vulgare* did not seem directly linked to lactic acid production; nevertheless, their presence might foster the growth of other bacteria.

The production of lactic acid during whey fermentation influences the occurrence of pathogenic genera, like *Clostridium* and *Salmonella*, which have a minimal presence (0.05–0.10%) due to their low acid tolerance in fermented whey. Notably, LAB prevails in spontaneously fermented whey, particularly when optimized for lactic acid formation (37–42 °C). This makes it an appealing resource for acidifying milk in cheese crafting. Furthermore, both fresh and fermented whey create a niche teeming with bacteria possessing probiotic and technological attributes.

Microbiological analysis and microscopic examination of the samples under study did not reveal the presence of foreign microflora within the whey samples. The microbial composition of the products primarily consists of yeast, lactobacilli, and lactococcal cultures. Additionally, four (4) yeast strains were successfully isolated from the whey samples for further examination.

The process of enriching yeast cultures relies on their capacity to thrive in sugar-containing substrates with a slightly acidic environment, as well as their resilience to ethyl alcohol. The yeast accumulation was assessed visually through the appearance of sediment and distinctive byproducts in the nutrient medium, as well as through microscopic examination. *Lactobacilli* were identified through microscopy.

To identify the species affiliation of yeast and LAB strains, a conventional approach involving the study of

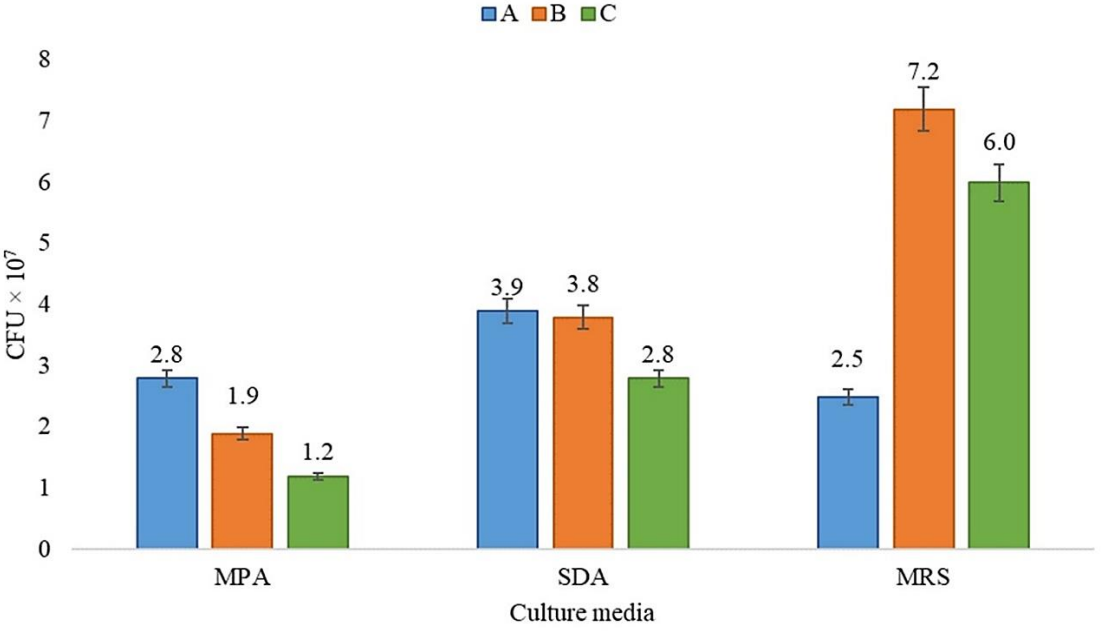


Fig. 2 Microbial taxonomic composition of research whey grown on various media.

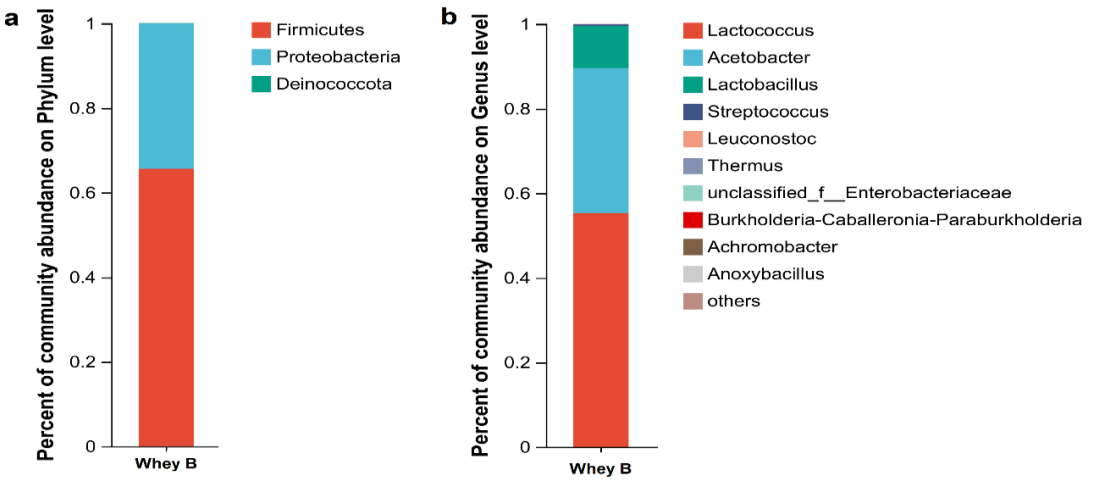
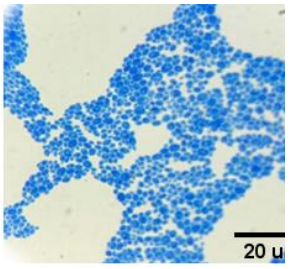
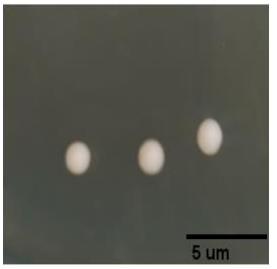
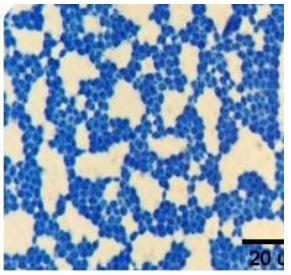
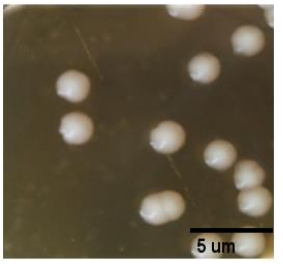
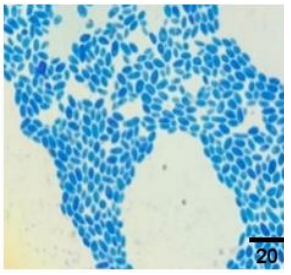
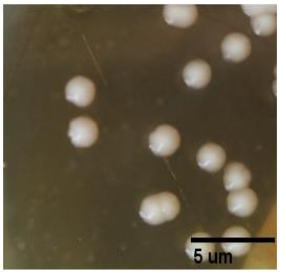
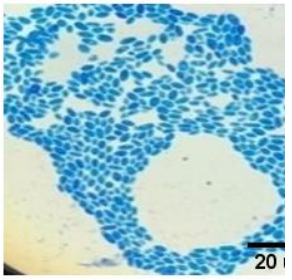
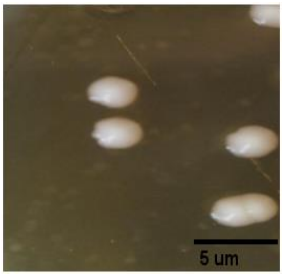


Fig. 3 Community abundance on phylum and genus levels in whey B.

Table 3. Yeast strains macro- and micromorphology.

Yeast strain	Macromorphology	Micromorphology
A1		
A2		
M1		
M2		

morphological and cultural characteristics was employed. The morphological and cultural attributes of the chosen yeast strains are outlined in Table 3.

The yeast strains exhibit round, medium-sized colonies on the solid medium's surface. These colonies possess a glossy, white appearance and a curd-like texture. The yeast strains reproduce through budding as part of their vegetative propagation method. The yeast cell dimensions range from 1.5 to 6.1 µm.

3.3 Isolation and identification of lactose-fermenting yeast strains

Yeast identification was conducted by analyzing the direct nucleotide sequence of the *ITS* region[54] followed by comparing the nucleotide identity with sequences stored in the international GeneBank database. DNA concentrations were obtained using the Qubit 2.0 fluorometer (Fig. 4).

After amplification using *ITS* primers (Table S1), PCR products with a size of approximately 750 bp were generated. Following the sequencing process, a secondary purification of the PCR product was conducted, and the resulting material was introduced into an ABI 3500 genetic analyzer for capillary electrophoresis.

According to the obtained results (Fig. 5), A1 and A2 yeast strains belonged to *Kluyveromyces marxianus*, M1 and M2 – to *Saccharomyces cerevisiae* with affinity levels of 100, 100, 100, and 99.86 %, respectively.

3.4 Yeast cells biochemical activity in whey

Whey serves as a comprehensive raw material for synthesizing protein substances, given its notable content of carbohydrates (lactose), mineral salts, and vitamins. Yeast stands out as a prime protein producer within whey, benefiting from its ability to utilize lactose as a nutritional source. On average, yeast in its absolutely dry form (ADF) comprises around 45–50% nitrogenous compounds, 2–5% fat, 25-35% carbohydrates, and 6-8% ash.[5] These nitrogen-containing compounds are biologically complete, encompassing proteins (70%), amino

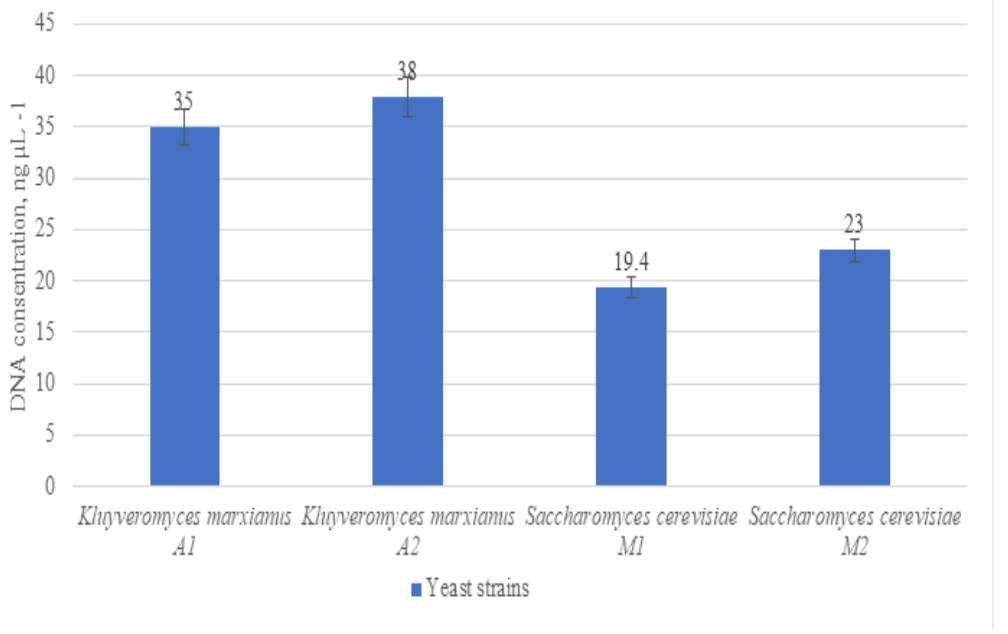


Fig. 4 DNA concentrations in isolated yeast strains.

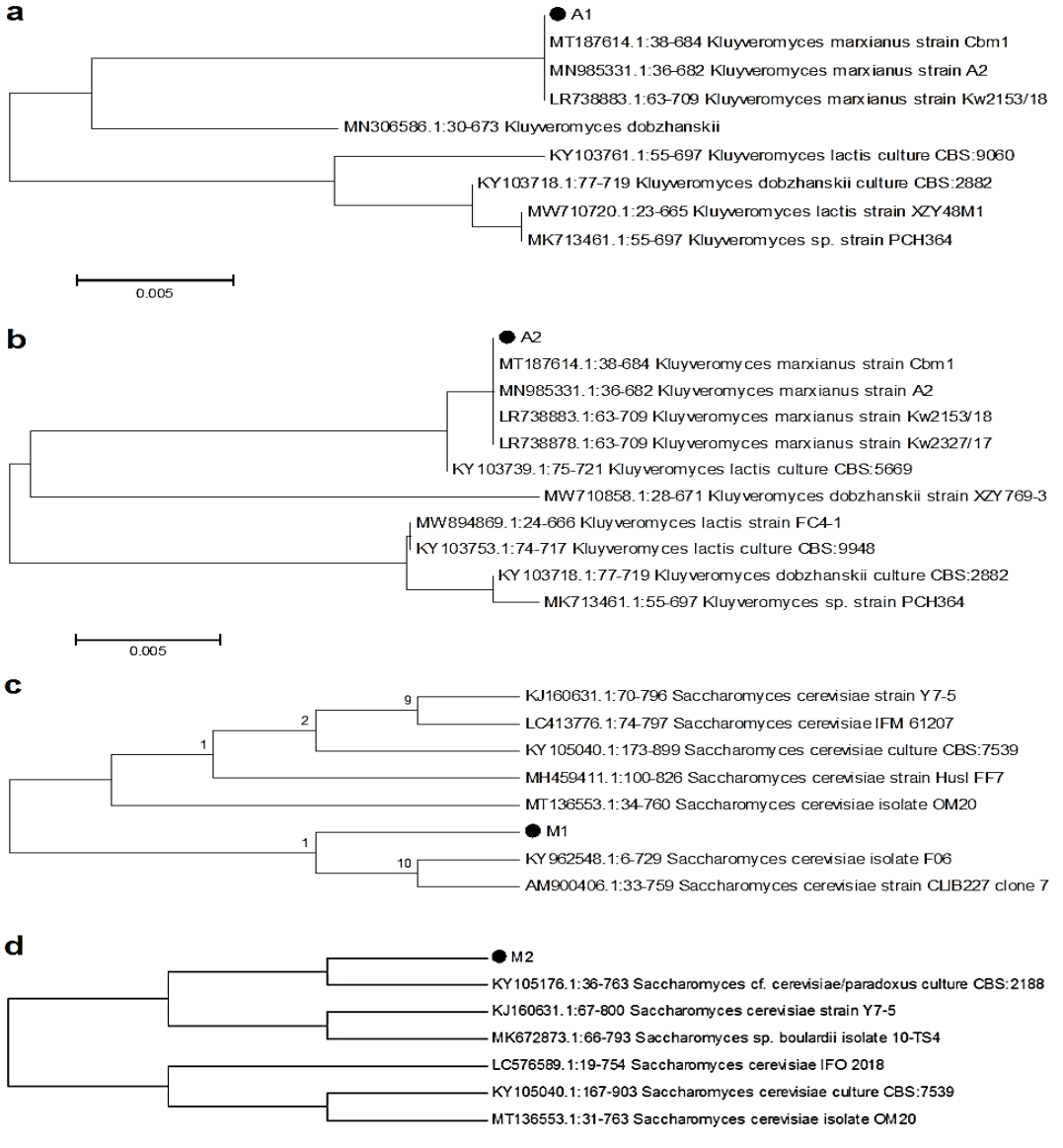


Fig. 5 Phylogenetic trees of the research yeast strains.

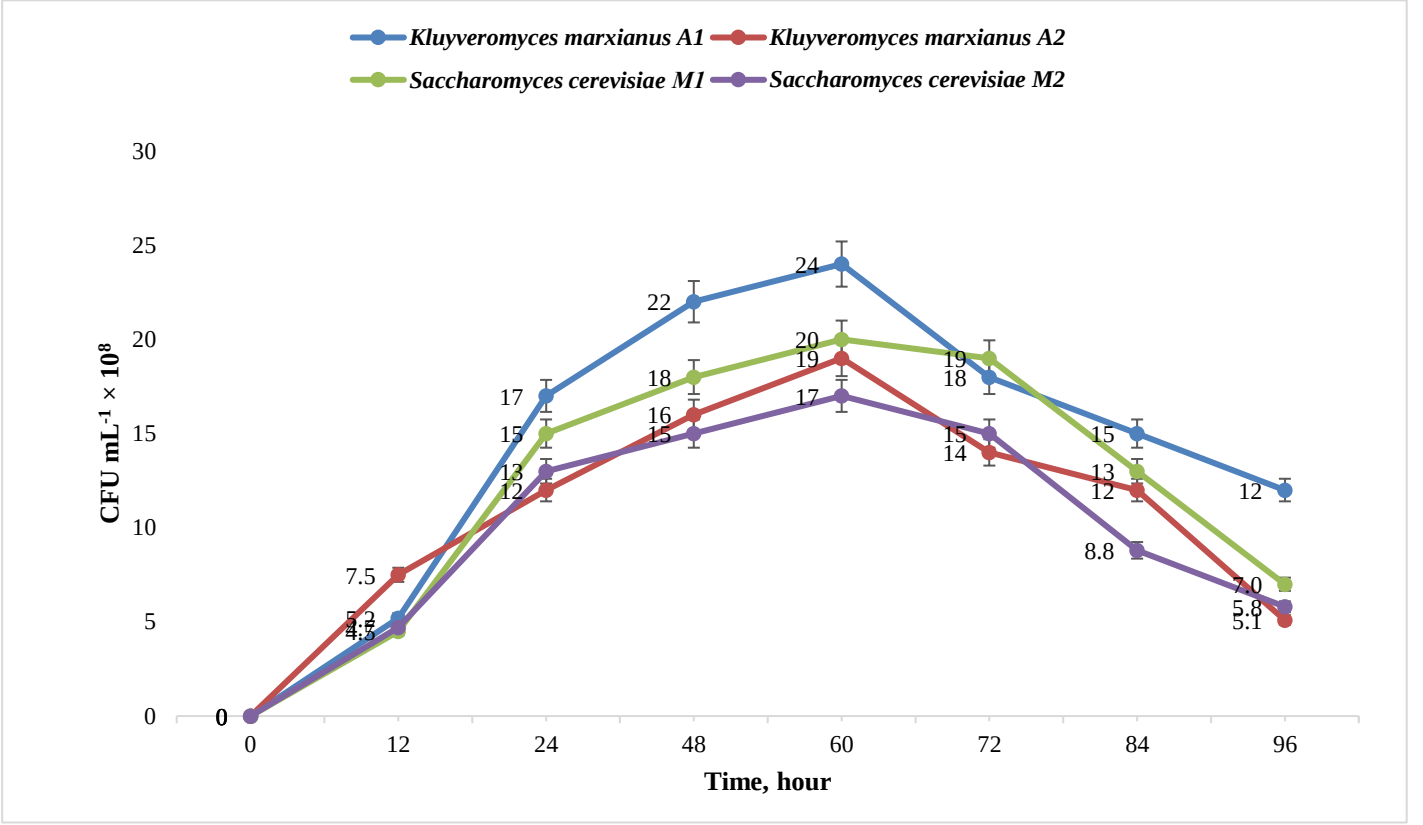


Fig. 6 Yeast cultures growth dynamics on whey.

acids, and nucleotides (15%), along with purine (8–10%) and pyrimidine (4%) bases. The dynamics of yeast growth on research whey samples is shown in Fig. 6.

The examination of yeast culture's growth dynamics on whey revealed a pronounced intensity of biomass accumulation across all strains. Notably, *Kluyveromyces marxianus* A1 exhibited particularly robust biochemical activity in connection with whey, with its cell count reaching 24×10^8 CFU mL^{-1}. In comparison, *Kluyveromyces marxianus* A2 displayed a cell count ranging from 18 to 20×10^8 CFU mL^{-1}.

Yeast exhibits the highest whey-to-microbial protein conversion rate. Specifically, the yeast *Kluyveromyces marxianus* possesses the capability to utilize lactose and inulin as carbon sources, producing notably active β-fructofuranosidase. This yeast displays a rapid growth rate and extensive exploitation in biotechnological processes to yield protein, being consistent with previously reported data.[12,18,55] During growth, yeast utilizes whey lactose and lactic acid as energy sources, converting mineral nitrogen-containing salts into comprehensive cellular protein. Yeast-derived whey significantly surpasses the original whey in terms of protein content. The presence of easily digestible carbon sources within whey designates it as a promising raw material. Additionally, the challenge of effectively utilizing whey holds significance from both economic and environmental perspectives.

Thus, among various whey processing methods, whey fermentation with diverse lactose-fermenting yeast cultures to yield alcohol remains a pertinent and particularly intriguing avenue for scientists.

3.5 Yeast cell immobilization on different carriers

The sugar fermentation process for ethanol production was conducted utilizing *Saccharomyces cerevisiae* cells immobilized within Ca-alginate films.[24,28] Rather than the conventional spherical bead configuration, thin Ca-alginate films deposited on the microchannel surface were employed. The immobilization of yeast on alginate films led to a higher ethanol yield compared to free yeast cells operating under identical fermentation conditions.

Various polymeric cryogels were employed for the immobilization of isolated yeast cultures, including PVA,[29,56-58] k-carrageenan,[26,59,60] and Na-alginate beads.[61] The yeast strains investigated encompassed *Kluyveromyces marxianus* A1, *Kluyveromyces marxianus* A2, *Saccharomyces cerevisiae* M1, and *Saccharomyces cerevisiae* M2.

The investigated yeast strains displayed significant sorption activity on PVA, ranging from 66 to 85% within 24 hours (Fig. 7). Particularly, *Kluyveromyces marxianus* A1 exhibited the highest sorption rate among the studied strains.

Immobilizing yeast cells on k-carrageenan revealed the following trends: *Kluyveromyces marxianus* A1 strain exhibited the highest sorption activity, reaching 70-85% after 1 h (Figs. 8 and S1). Both *Saccharomyces cerevisiae* M1 and *Saccharomyces cerevisiae* M2 strains demonstrated equal sorption rates that gradually increased after 0.5 h of immobilization, ranging from 42 to 68%. *Kluyveromyces*

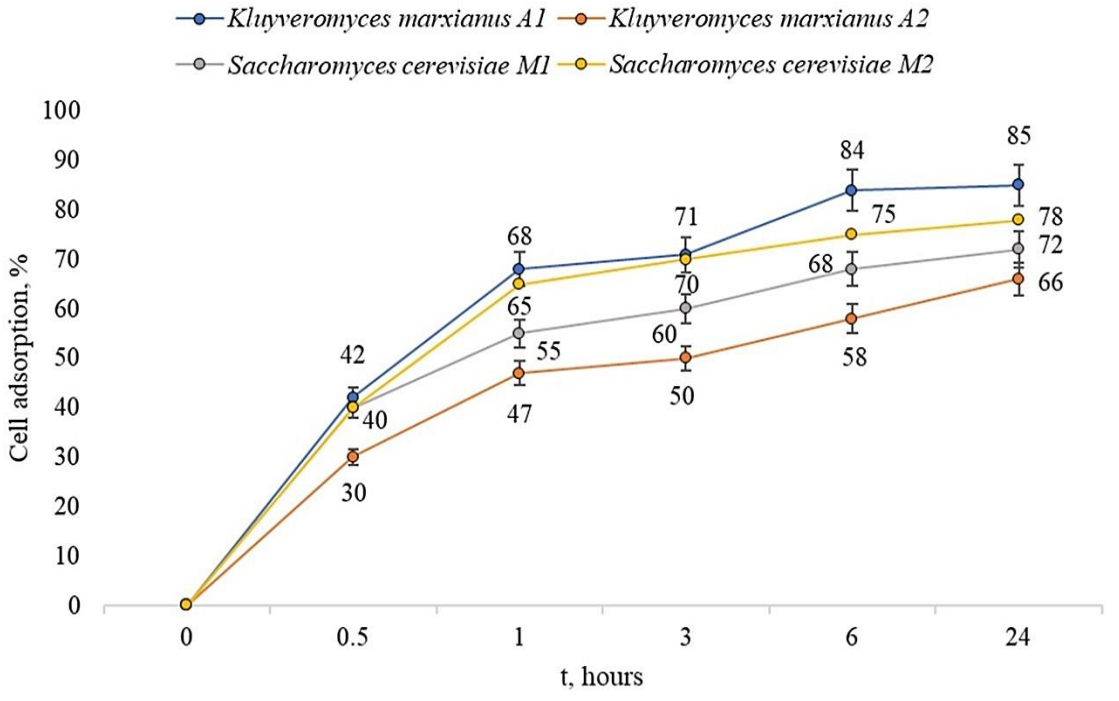


Fig. 7 Yeast cell adsorption on PVA.

marxianus A2 strain displayed the weakest sorption activity, reaching only 47% within a 24-hour span (Fig. 8).

The immobilization of yeast cells on Na-alginate beads followed a similar pattern as observed during immobilization on k-carrageenan, with one exception for *Kluyveromyces marxianus* A2. Specifically, *Kluyveromyces marxianus* A1 strain showcased the highest sorption activity (58-82%; Figs. 9 and S2). *Saccharomyces cerevisiae* M1 and *Saccharomyces cerevisiae* M2 strains exhibited comparable sorption rates (38-72%). *Kluyveromyces marxianus* A2 initially displayed a slightly lower sorption rate within the first 6 hours after immobilization, but within 24 hours, its sorption rate aligned with that of *Saccharomyces cerevisiae* M1and *Saccharomyces cerevisiae* M2, reaching 68% (Fig. 9).

Thus, using various carrier materials for yeast cell immobilization demonstrated that the *Kluyveromyces marxianus* A1 strain holds the most potential for further exploration of its bioethanol production capabilities.

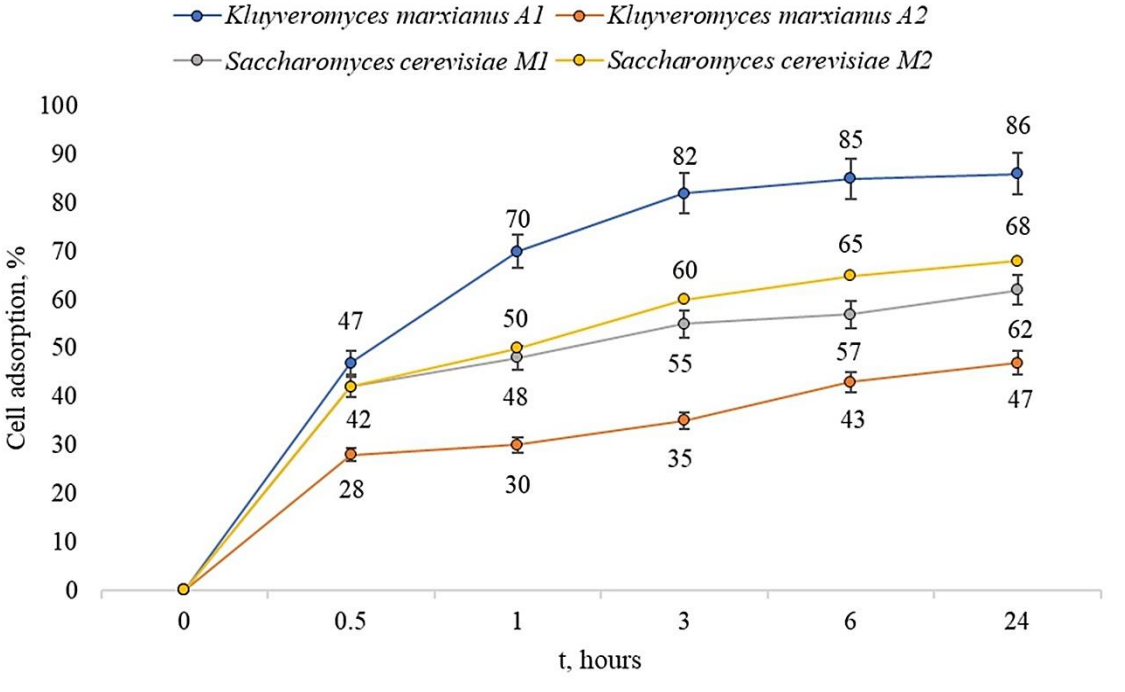


Fig. 8 Yeast cell adsorption on k-carrageenan.

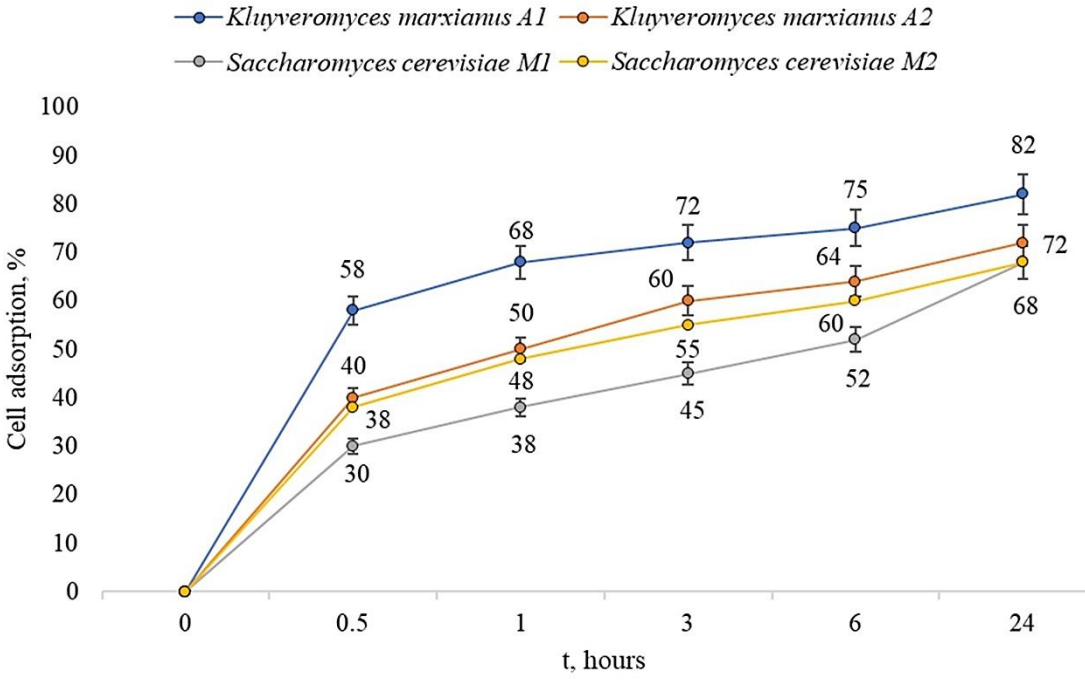


Fig. 9 Yeast cell adsorption on Na-alginate beads.

3.8 Bioethanol production by immobilized yeast cells

The transition from free microbial cells to biocatalysts is a significant advancement. While bioethanol production from free yeast cultures already yields relatively high amounts where immobilizing yeast cultures is anticipated to further enhance bioethanol yield.

Bioethanol accumulation capacity of *Kluyveromyces marxianus* A1 and *Kluyveromyces marxianus* A2 cells immobilized on k-carrageenan and Na-alginate beads in the whey was measured for 24-, 48-, and 96-hour periods (Fig. 10). *Kluyveromyces marxianus* A1 demonstrated a notably higher bioethanol production capacity in comparison to *Kluyveromyces marxianus* A2 (Fig. 10). A key distinction between the studied strains lies in the potential for bioethanol yield: *Kluyveromyces marxianus* A1 has the possibility of achieving even higher yields in the subsequent days, while *Kluyveromyces marxianus* A2 strain peaked on the 2nd day with no further potential for increased yield. Comparing the bioethanol yield in *Kluyveromyces marxianus* A1 cells, it can be deduced that within 48 h, their production capacities were comparable. However, during the subsequent 48 h, a significant difference emerged: the bioethanol yield in cells immobilized on k-carrageenan was nearly double (1.76 times higher) in comparison to the yield in cells immobilized on Na-alginate beads.

Since k-carrageenan served as the best carrier material for bioethanol production, it was decided to analyze the lactose concentration in *Kluyveromyces marxianus* A1 and

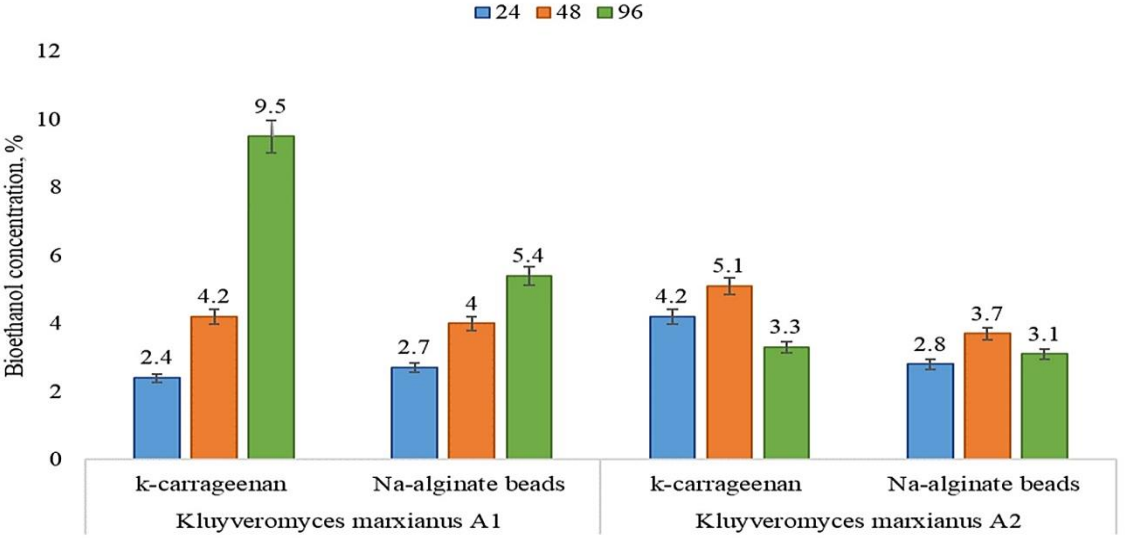


Fig. 10 Bioethanol yield produced by yeast cells immobilized on k-carrageenan and Na-alginate beads.

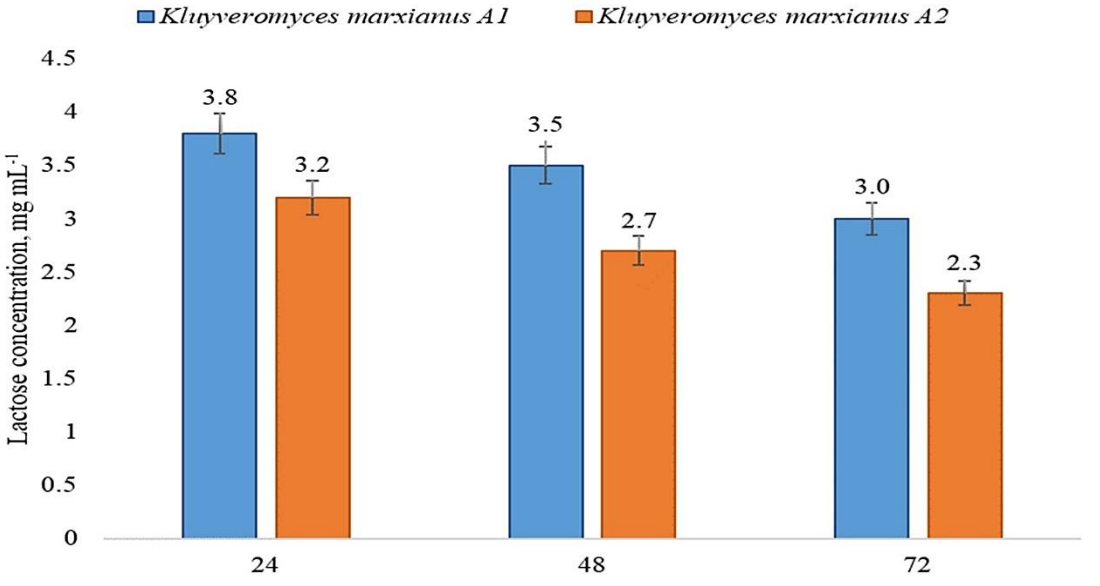


Fig. 11 Lactose concentration in yeast cells immobilized on k-carrageenan.

Kluyveromyces marxianus A2 cells immobilized on this carrier (Fig. 11).

Both yeast cells shared one trend of decreasing the lactose content within 72 h; however, initially, *Kluyveromyces marxianus* A2 cells contained a lower amount of lactose.

4. Conclusion

The research findings yield several significant conclusions: technical whey exhibited comparable and, in some cases, superior attributes for utilization in bioethanol production. *Firmicutes, Proteobacteria, Actinobacteria*, and *Bacteroidetes* are key microbial groups in whey, regardless of fermentation temperatures. *Kluyveromyces marxianus* A1 cells exhibited superior and uniform adsorption rates (82-85%) across all three cost-effective carrier materials amongst the studied carriers when compared to other tested strains. The immobilization of *Kluyveromyces marxianus* A1 on k-carrageenan for 96 hours yielded the highest bioethanol production, with the potential for further enhancement. Future investigations on optimizing whey-to-bioethanol valorization via yeast cell immobilization are required for industrial-scale production.

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Conflict of Interest

There is no conflict of interest.

Supporting Information

Applicable.

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