



Valorization of Cheese Whey through Bioethanol Production and Probiotic Drink Development: A Sustainable Approach for Organic Waste Management

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Abstract

Cheese whey, a significant byproduct of the dairy industry, poses a substantial environmental challenge due to its organic pollutant load and large size demands for effective and affordable valorization methods. The abundance of lactose and other organic compounds in whey contributes to its elevated Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD), further straining natural ecosystems. This study aimed to investigate the nutritional potential of whey and its conversion into value-added products: bioethanol and probiotic drinks. To achieve this, *Bacillus megaterium* and *Bacillus subtilis* strains, isolated from soil samples in Nepal, were co-cultured with *Lactobacillus* sp. and *Saccharomyces cerevisiae* in the whey broth for fermentation. Ethanol distillation was done using a rotary evaporator to maintain the viability of the fermenting organisms in the broth. Moreover, the probiotic criteria of the fermenting strains were extensively examined. Experimental observations revealed a remarkable concentration of 9.2 g/L of ethanol, resulting in an ethanol yield of 0.23 g/L (42% compared to theoretical yield). Extrapolating this rate, it is theoretically possible to produce an annual global bioethanol output of 5 million tons using whey as a sustainable and abundant resource. Furthermore, the study explores novel advancements in the distillation process, demonstrating the successful extraction of ethanol at room temperature. Additionally, the fermentation organisms employed in this research exhibit robust viability, surviving various in-vitro stress-tolerance tests, including temperature, bile salt, pH fluctuations, and gastric juice. These resilient strains also demonstrated long-term stability in fermented whey broth, making them promising candidates for the development of probiotic beverages. This dual approach enables sustainable management of organic waste and presents an opportunity to create value-added products with positive implications for the environment and human health.

Keywords: Whey, Valorization, Fermentation, Bio-ethanol production, Probiotic drinks, In-vitro tolerance

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Introduction

Cattle farming, as one of the oldest and most widely practiced agricultural occupations worldwide, serves as a sustainable means of milk production. This industry's main function is to transfer money from wealthier to impoverished communities through milk production, a vital source of nutrition. However, the high perishability and water content (85-90%) of raw milk present challenges in its transportation, resulting in only 8% of total raw milk being transported without undergoing processing [1]. Cheese, a leading milk product, plays a crucial role in long-distance dairy commerce due to its superior food value and extended shelf life compared to raw milk. The production process involves coagulating milk using suitable agents such as vinegar or lemon, etc. [2]. Globally, approximately 35% of raw milk is converted into cheese, making it a significant contributor to the dairy industry [3]. Cheese production not only addresses

the issue of surplus milk during certain seasons but also helps overcome "milk holidays" when milk procurement from farmers is interrupted. Furthermore, cheese production offers a solution to address lactose intolerance problems, as the fermentation process reduces lactose content, making it more tolerable for individuals with lactose intolerance [4].

However, the production of cheese results in a substantial byproduct known as cheese whey. Theoretically, for every kilogram of good cheese produced, approximately nine kilograms of whey are left over [5]. In 2020 alone, over 16 million metric tons of cheese were produced worldwide, suggesting that a significant amount of whey, approximately 9 x 16 million metric tons, was generated [6]. Whey is rich in essential nutrients, representing approximately 55% of the total milk nutrition and containing components such as lactose, soluble salts, globular proteins, trace fats, and

vitamins. Lactose, which accounts for about 70% of milk lactose, contributes to the high Biological Oxygen Demand (BOD); 30,000 mg/L and Chemical Oxygen Demand (COD) ; 70,000 mg/L levels in whey [7]. The disposal of large quantities of whey poses a significant environmental challenge. It has been estimated that a small cheese factory producing 4,000 liters of whey per day (equivalent to 450 kg of cheese) would generate as much sewage as 1,900 people [8]. The high nutrient content of whey presents a threat to both cheesemakers and the ecosystem at large, highlighting the need for effective technology to trap and utilize its organic compounds. Various approaches are being explored for the valorization of whey lactose, with microbial fermentation being a promising route for producing value-added products, such as bioethanol.

Fermenting lactose in whey presents several challenges. Cells must possess a *Lac* operon system, producing β -galactosidase and galactosidase permease, to metabolize lactose effectively [9]. Only specific microorganisms, such as lactic acid bacteria (LAB) and certain wild yeast strains like *Kluyveromyces lactis*, *K. marxianus*, and *Candida pseudotropicalis*, have this system, but they exhibit sensitivity to ethanol concentration and have relatively low sugar conversion rates, making them less than ideal for lactose fermentation [10] and [11].

Saccharomyces cerevisiae, a genetically recognized as safe (GRAS) yeast species, is widely used for industrial fermentation. It has properties such as high ethanol tolerance (up to 20%), pH tolerance (up to 3.0), high yield, short generation time, and superior fermentation capacity compared to direct lactose fermenting organisms [12] and [13]; [14]. Although *S. cerevisiae* lacks the *Lac* operon system, genetic manipulation of the *Lac* gene or the hydrolysis of lactose into its monomers glucose and galactose, followed by fermentation by the yeast, have been explored [12]. The enzyme β -galactosidase (E.C 3.2.1.23) is widely used in the food industry to produce lactose-free products. It breaks down the glycosidic bond of lactose, yielding glucose and galactose [15]. β -galactosidases are produced by various organisms, including plants, bacteria, and fungi, with microorganisms, particularly bacteria, being dominant producers due to their ease of fermentation, high enzymatic activity, and stability [16]; [17]. It varies with organisms whether this enzyme is produced both extracellularly or intracellularly. *Bacillus* species have been investigated for the extracellular production of β -galactosidase, which is safe for use in the food industry [18]. Lactic acid bacteria, including *Bacillus* sp. and

Lactobacillus sp., have a good capacity for lactose metabolism and are naturally associated with yeast on different substrates, fermenting together [15]. In such co-cultures, yeast dominates as the primary fermenter, converting reducing sugar into ethanol, while LAB produces different organoleptic organic compounds that prevent cross-contamination during fermentation [19]. This natural association between yeast and LAB has been explored to produce bioethanol using whey as a substrate.

In addition to bioethanol, Whey can also be used to produce probiotic drinks. Probiotics are live microorganisms that, when consumed adequately, provide health benefits to the host [20]. Lactic acid bacteria and certain yeasts, including *Saccharomyces*, *Kluyveromyces*, *Bacillus megaterium*, and *Bacillus subtilis*, have been identified as potential probiotics [21] and [22]. Probiotics have a range of benefits, including improved digestion, prevention of pathogen growth, and enhancement of bowel health and immunity [23] and [24]. Choosing appropriate probiotic candidates is crucial, considering their viability during production, storage, and exposure to the host environment, as well as their proven health benefits. *Saccharomyces cerevisiae* is commonly used in the food industry to improve gut microbiota, and it has been found to enhance digestion and have potential applications in sucrose deficiency [25]; [23] and [22].

Materials and method

Sample collection and characterization

For sample collection and characterization, polypropylene bottles were thoroughly rinsed with 2% caustic soda **followed by hot water for 2 times** to ensure cleanliness. Freshly prepared whey samples were then collected from 10 different cheese producers in Birgunj, Nepal. To remove any impurities, the whey samples were filtered through muslin cloth and subjected to pasteurization in the laboratory. Following pasteurization, the whey samples were stored in a refrigerator at -4°C until further use. Microbiological analysis was conducted to determine the microbiome profiles of the whey samples. Additionally, various parameters including pH, lactose content, protein content, phosphate, calcium, vitamin content, and biological oxygen demand were studied using established methods cited in relevant literature sources [26]; [27,28].

Isolation and validation of organisms

The isolation and validation of organisms involved the collection of soil samples from five different areas in the

Panchase region of the Pokhara valley of Nepal. Bacterial strains *Bacillus subtilis* and *Bacillus megaterium* were isolated and characterized. One gram of each soil sample was enriched in 5 ml of sodium acetate base media at pH 6.5 and subjected to heat treatment at 80°C for 10 minutes to eliminate vegetative cells. Pour plating techniques were employed using acetate agar media, and the plates were then incubated at 28°C for 48 hours. Subsequently, fast-growing large colonies of *B. megaterium* were transferred to L-tryptophan and phenol agar media for further screening. Chalky colonies indicative of presumptive *B. subtilis* were transferred to agar media containing malic acid, catechol, and phenol. Surviving colonies on these selective media were further characterized through morphological, biochemical, and molecular methods to validate putative *Bacillus* strains. Additionally, *Lactobacillus* (LB) and *Saccharomyces cerevisiae* (SC) strains were procured in activated dried powdered form and revived in MRS (De Man, Rogosa, and Sharpe) and SD (Sabouraud dextrose) broth, respectively.

Validating Lactose metabolizing nature of *Bacillus* strains

To validate the lactose-metabolizing nature of the *Bacillus* strains, the NA X-gall method was employed. This method assessed the ability of the bacterial strains to metabolize lactose. Furthermore, the β -galactosidase enzyme activity was determined using the ONPG test method, which followed the procedures described by Percival *et.al.*, 2019.

Whey fermentation

For whey fermentation, a 2-liter fermentation batch was prepared using whey (Composition: Na₂CO₃ -0.53 gm, NH₄Cl -0.30 gm, KCl -0.12gm, Whey 13%) as the substrate in a fermenter equipped with an airlock and thermometer. The initial pH was adjusted to 6.5 by adding sodium carbonate, and the mixture was subsequently autoclaved. An overnight bacterial culture comprising strains *Bacillus megaterium* (S13), *Bacillus subtilis* (S24), and *Lactobacillus* (LB), with an inoculum size of 1-2% relative to the batch size, was aseptically inoculated into the fermenter. The fermentation process took place at room temperature (28 ± 2°C) for a duration of 24 hours. Simultaneously, a yeast inoculum of 200 ml was prepared in Sabouraud dextrose broth (SDB) using an overnight culture of *Saccharomyces cerevisiae*. On the following day, the yeast inoculum and yeast nutrients were aseptically transferred to the fermenter to facilitate fermentation under optimal conditions (temperature 28 ± 2°C and humidity 55±5%).

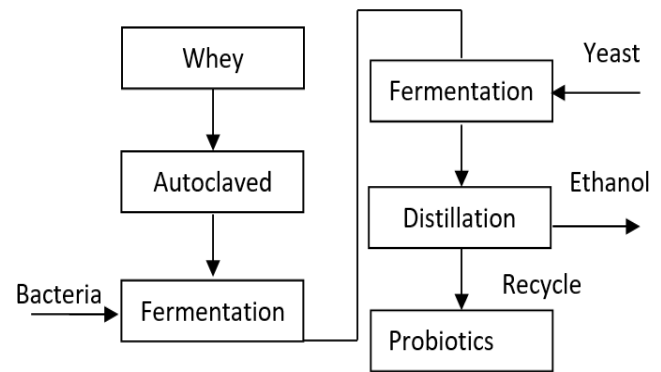


Figure 1. Graphical representation of valorization of whey with co-culture of yeast and LAB (modified from Mawson, 1994)

The pH change, lactose concentration, and ethanol concentration were monitored and measured at the end of the fermentation, by using methods described by Seo *et.al.*, 2009. After fermentation, ethanol distillation was then performed using both traditional and revised methods. In conventional distillation type ethanol was recovered by evaporation followed with condensation at high temperature whereas in revised method we tried to collect ethanol from broth under vacuum pressure using rotary evaporator instead of high temperature. Moreover, the remaining broth after ethanol distillation was subjected to a viability test using the pour-plate method in a specific medium. This test aimed to determine the presence and viability of the fermenting organisms in the remaining broth.

Probiotic criterion tests

For probiotic criterion tests, several in-vitro assessments were conducted to evaluate the presumptive probiotic strength of the organisms used in fermentation. Temperature, pH, bile, and gastric juice tolerance tests were performed to determine the organisms' ability to withstand and survive under simulated gastrointestinal conditions. Following these tests, the fermentation broth that remained after ethanol distillation was placed in a stability chamber at room temperature (RT) and relative room humidity (RH) for one month. After the incubation period, a cell viability test was conducted using the pour-plate method with a 100-fold dilution to assess the survival and viability of the organisms in the broth. To examine the potential cross-contamination with pathogens, a pathogenic test was carried out using WHO prioritized strains, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella*, and *Shigella*.

Results and discussion

Whey samples collection and characterization

The whey sample collection and characterization revealed important insights regarding the current practices and composition of whey in the cheese manufacturing industry. Field observations indicated a lack of awareness among cheese manufacturers regarding the nutritional value of whey and the absence of effective utilization strategies. It was commonly observed that whey was considered a valueless byproduct and disposed of untreated. The whey samples were subjected to detailed analysis, and the average results are summarized in the table below.

Table 1. Composition of the whey samples analyzed

Components	Composition of Whey
Color	White or golden yellow
pH	4.5
Total solid	55g/L
Total protein	9.8g/L
Lactose	41g/L
Soluble Phosphate	45.9 µg/L
Soluble calcium	0.39g/L
Vitamin B ₂	0.0016 mg/L
Vitamin B ₆	0.0098 mg/L
BOD	5500 mg/L
Total calorific value	32 Kcal/L

*All the values are the average data of 10 whey samples studied.

The findings indicated that approximately 70-80% of the milk volume is eluted as whey during cheese production. The major organic compound present in whey was lactose, accounting for around 4.1% of the composition. Protein content was found to be approximately 0.98%, with trace amounts of phosphate, calcium, and vitamins also present. These composition findings were consistent with previous studies, such as the work conducted by [30]. The pH of the whey samples ranged from 3.5 to 7.3, which was influenced by the source of milk and the type of cheese coagulation methods employed. This variation in pH is well-documented in the research conducted by [31]. Biological oxygen demand (BOD) analysis revealed that the whey samples required 5500 mg/L of oxygen for breakdown, which was significantly lower than the values reported in the review by [32]. This discrepancy may be attributed to in-process errors or sample variations.

Considering the current global dependence on non-renewable petroleum-based energy sources and their detrimental impact on climate change, researchers are actively exploring alternative substrates to produce green energy, such as bioethanol. However, the use of grains and sugarcane, which are primarily cultivated for food markets, as fermentation substrates for bio-ethanol

production remains a topic of debate. Therefore, whey lactose emerges as a potential alternative substrate for bio-ethanol production due to its abundance, low cost, easy availability, and the presence of essential minerals and vitamins necessary for microbial manipulation. This utilization of whey lactose as a renewable and sustainable source of energy aligns with the global efforts to reduce reliance on fossil fuels, as highlighted by [33] and [34].

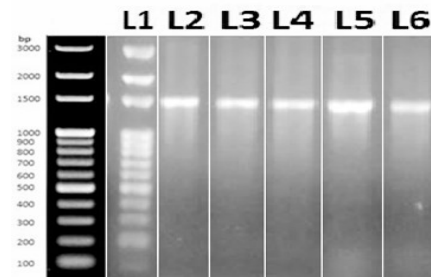


Figure 2. PCR amplification of *Bacillus megaterium* genomic DNA by universal 16s rRNA primer. Ladder used is of GeneRuler™ 100 bp which is supplied with 6X loading Dye on 1% agarose gel electrophoresis. (L1=100 bp ladder, L2= Amplicon from S13, L3= Amplicon from S15, L4= Amplicon from S23, L5= Amplicon from S28, L6= Amplicon from S32.)

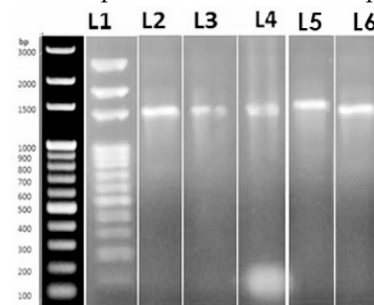


Figure-3: PCR amplification of *Bacillus subtilis* DNA by 16s rRNA universal primer. Ladder used is of GeneRuler™ 100 bp which is supplied with 6X loading Dye. L1=ladder, L2= Amplicon from S1, L3=Amplicon from S2, L4= Amplicon from S8, L5= Amplicon from S21, L6= Amplicon from S24

Organisms Isolation and validation

Bacillus megaterium and *Bacillus subtilis*

Bacillus strains were isolated from the soil samples as explained by [35]. These strains are endospore-forming which make it easy to select cells under high temperatures and by using selective carbon media as studied in [36]. The colonies exhibited viability on tryptophan and phenol media, indicating that they were putative *Bacillus megaterium* strains. Whereas colonies isolated in malic acid followed by catechol and phenol were presumed to be *Bacillus subtilis* strains. These findings align with the previous work conducted by [37] and [38], and further support the identification of these colonies as *Bacillus megaterium* strains. These presumptive isolates were further validated through microscopic analysis followed with biochemical studies which revealed that only nine isolates of *B. megaterium* and 17 isolates of *B. subtilis* exhibited Gram-positive

characteristics and rod-shaped morphology, positive reactions for catalase and citrate utilization, while indole production was negative. These positive biochemical characteristics narrowed down the selection to nine isolates, which were then subjected to further molecular tests. Through the findings of [39], we can consider these strains were possibly the *Bacillus* strains we are targeting. To conduct molecular characterization, genomic DNA (gDNA) was extracted from the isolates. PCR amplification was performed using universal primers targeting the 16s rRNA genomic region. Gel electrophoresis of the PCR products revealed similar band sizes in five samples, while a 100 bp ladder was used as a size reference. The amplified product exhibited a size of 1500 bp, confirming bacteria' presence and suggesting that these isolates were putative *B. megaterium* strains and *B. subtilis*. This molecular characterization provided additional evidence to support the identification of these isolates as *Bacillus megaterium* strains and laid the foundation for further investigations and analysis in the study.

The sequence obtained by DNA sequencing was further characterized by multiple sequence alignment. The highly similar sequence obtained in BLAST search indicates a significant alignment of the putative DNA sequence to 16s rRNA genes of *Bacillus megaterium*. The cluster approach called neighbor joining (NJ) method was used for the reconstruction of phylogenetic trees that yielded unrooted tree with branch lengths as follows:

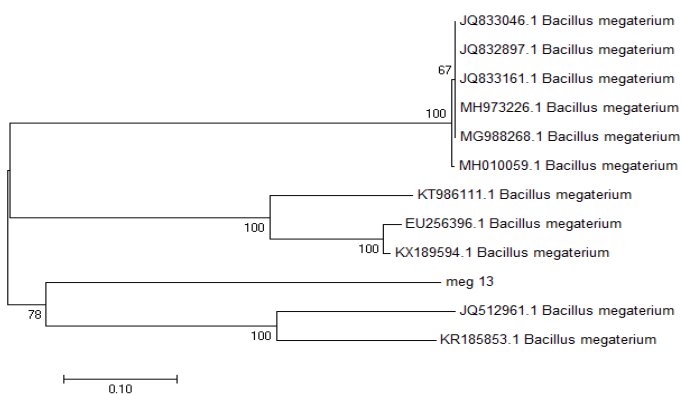


Figure 4. 16s rRNA sequence tree generated with the neighbor joining method with 1000 bootstrap resampling for *Bacillus megaterium* (S13).

According to the 16s rRNA phylogenetic tree, S13 has a close relationship with *Bacillus megaterium*. The tree shows that the 16s rRNA sequence of this organism is very similar to *Bacillus megaterium* 16s rRNA sequences. The bootstrap results in common interprets the probability that phylogenetic estimation represents the true phylogeny. Explicitly, under various conditions such as equal rates of change, symmetric phylogenies, and

internodal change of <20% of the characters, bootstrap proportions of >70% usually correspond to a probability of >95% that the corresponding clade is real. Therefore, S13 showed identical similarities with *Bacillus megaterium*.

Furthermore, the obtained DNA sequences of *B. subtilis* were analyzed through multiple sequence alignments and compared to known sequences in the BLAST database. The results of the BLAST search revealed a significant alignment between the putative DNA sequence and the 16S rRNA genes of *Bacillus subtilis*, further supporting their identification. Phylogenetic trees showed evolutionary relationships among the putative *Bacillus subtilis* strains and other related organisms.

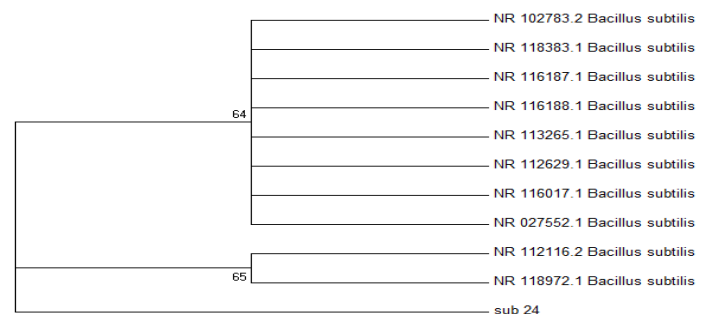


Figure 5. 16s rRNA sequence tree generated with the neighbor joining method with 1000 bootstrap resampling for *Bacillus subtilis* 24.

In the 16S rRNA phylogenetic tree, strain S24 showed a close relationship with *Bacillus subtilis*, indicating a high similarity between their 16S rRNA sequences. Based on these analysis, strains S13 (*Bacillus megaterium*) and S24 (*Bacillus subtilis*) were selected for further studies.

The selection of these bacterial strains was based on their initial potential as probiotics and their ability to utilize lactose as a carbon source. Additionally, yeast strains were selected for their ability to ferment sugar and produce bioethanol, while also possessing probiotic capabilities. Previous studies, such as the one conducted by [15], have demonstrated the lactase synthesizing ability of *Bacillus* species, making them suitable for lactose sugar hydrolysis.

Validation of bacteria's lactose metabolizing nature

Blue colonies of strains S13 and S24 were observed on the nutrient agar plates infused with X-gal which indicates the presence of the hydrolyzing enzyme β -galactosidase. Nutrient agar medium supplemented with X-Gal, a chromogenic substrate, was used for confirming the β -galactosidase producing organisms [40]. However, colonies of *S. cerevisiae* were observed white in color which resembled the color of control indicates the absence of lac operon in their cells.

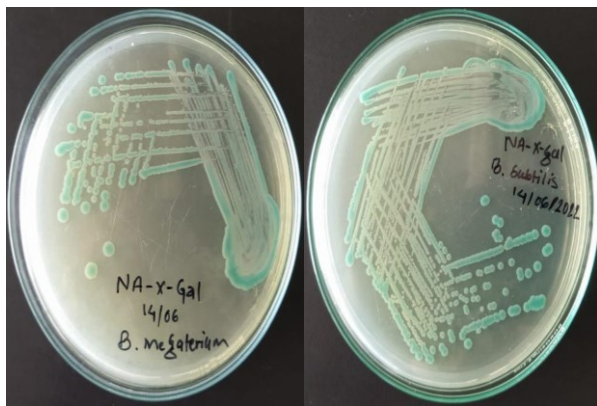


Figure 6. Blue colonies of bacteria on NA plates infused with X-Gal.

Growth of *B. megaterium* (S13), *B. subtilis* (S24) and *Lactobacillus* sp. in whey

To investigate the growth behavior of the selected bacterial strains in whey, a medium with whey as the sole carbon substrate was prepared. The optical density (OD) of the cultures was measured at 600nm at 24-hour intervals and the data was plotted on a graph. The results revealed that all three bacterial strains exhibited healthy growth in the whey medium.

Among the three strains, LB showed the maximum growth in the whey medium. This observation could be attributed to the adaptation of LB to dairy products, as it is commonly found in such environments. On the other hand, strains S13 and S24 are soil-borne bacteria, and their ability to metabolize lactose in the whey medium requires further investigation and study. Interestingly, when all three bacterial strains were combined in a consortium, their growth pattern was significantly higher compared to the individual organisms. This suggests that the interaction and synergistic effects between the strains may have contributed to the enhanced growth in the whey medium.

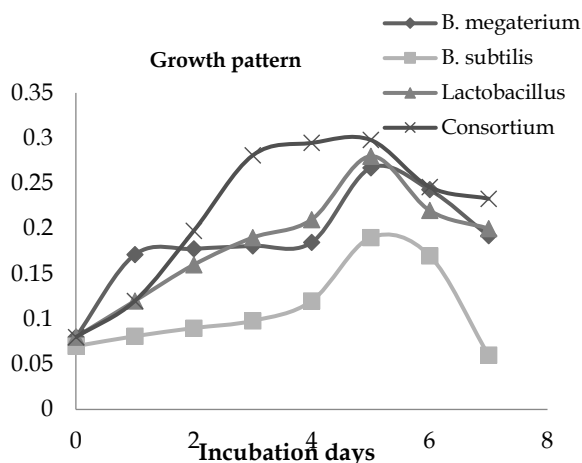


Figure 7. Curve showing growth of organisms in whey broth, lactose as sole available carbon source.

The growth data obtained from the OD measurements and plotted graph provide valuable insights into the growth dynamics of the bacterial strains in the whey medium. Further investigations and detailed studies are required to explore the lactose-metabolizing capabilities of strains S13 and S24, as well as to understand the specific mechanisms behind the observed growth patterns in the consortium.

Fermentation of whey lactose

After fermentation, a quantitative analysis of the fermentation broth was conducted. The summary of whey wine fermentation was given in the table.

Table 2. Summary of whey fermentation (after 9 days)

S.N.	Parameter	Result	
		Before fermentation	After fermentation
1.	°Brix	5.5	1.6
2.	Color	Clear yellowish	Turbid white
3.	pH	3.8	3.5
4.	Lactose	41g/L	12g/L
5.	Ethanol	-	9.2g/L
6.	Ethanol yield	-	0.23g/L
7.	BOD	5500 mg O ₂ per liter	1200 mg O ₂ per liter

The ethanol concentration in the whey wine samples was determined using the method described in [41], revealing an ethanol concentration of approximately 0.92% with a sugar conversion rate of 72% at the end of the fermentation after 9 days. It is worth noting that this ethanol concentration is relatively low compared to other whey fermentation studies. For instance [42] reported an ethanol concentration of 7.4% using a genetically engineered strain of *Saccharomyces cerevisiae*. The lower ethanol concentration observed in this study may be attributed to factors such as the relatively low lactose concentration, competition for substrate among different organisms, and the slow rate of fermentation.

Despite the lower ethanol concentration achieved, it is worth considering the potential impact on the world fuel market. The 72% yield obtained in this study suggests that, in the year 2020 alone, around 5 million tons of ethanol could have been produced worldwide if the entire lactose production was converted [6]. This represents a significant contribution towards addressing the global energy crisis and the search for alternative fuels to replace fossil fuels.

Additionally, the calculation of the biochemical oxygen demand (BOD) of the fermentation broth indicated a reduction in the organic molecule load of approximately 80%. This reduction can be attributed to the microbial activities involved in converting lactose into simpler

molecules. Consequently, this fermentation method offers potential environmental benefits by alleviating the issues associated with whey management and reducing the environmental impact.

While the observed ethanol concentration may not make whey lactose an optimal choice as a fermentation substrate for bio-ethanol production compared to recognized substrates like sugarcane and cornstarch, or emerging second-generation technologies utilizing lingo-cellulosic biomass [12], it is important to consider the advantages of utilizing whey as a waste effluent. Whey represents a significant volume of waste, providing a distinct advantage over food feedstock such as corn for ethanol production. Furthermore, feedstock costs constitute a major portion of overall biofuel production costs, with two-thirds of ethanol production costs attributed solely to the substrate [43]. The exploitation of whey lactose as a fermentation substrate could potentially alleviate the growing conflict between food and fuel production, particularly considering the projected increase in global food demand. According to a report by the World Bank, the world will need 70 to 100% more food by 2050 due to population growth [44]. Therefore, utilizing whey lactose as a substrate offers a promising solution to this conflict. Additionally, unlike fossil fuels that take millions of years to form, whey can be generated sustainably within a relatively short period alongside cheese production. Furthermore, the abundant availability of bio-waste is not only characterized by quantity but also by renewability, which are significant advantages over fossil fuels. Moreover, milk production resources are more evenly distributed geographically compared to fossil fuel reserves.

Distillation

Following the analysis of the whey wine, ethanol was distilled from the fermentation broth using different temperature conditions. The results and conditions of the distillation process are summarized in the table below.

Table-3: Summary of alcohol distillation.

S.N.	Parameters	Distillation conditions / results	
		Conventional (applying high heat)	Using rotary evaporator
1.	Temperature (°C)	80-90	28-30
2.	Vacuum	-	applied
3.	Time consumed (for 1 liter mead)	20-25 minutes	More than 2 hrs.
4.	Condition of whey wine	Darker yellow	No change
5.	Cells viability	Nil	Viable

It was observed that ethanol could be successfully distilled at various temperatures, with the fermentation broth remaining clear in color and the viability of the organisms unaffected when distillation was performed at room temperature. However, at higher temperatures, a dark coloration of the broth was observed. This discoloration may be attributed to reactions between residual sugars present in the broth and nitrogen compounds, which can lead to damage and make the management of the spent wash challenging [45]. Additionally, it is important to note that as the ethanol concentration in the broth decreases, the rate of energy consumption during distillation increases, which is a drawback of conventional distillation processes used in microbial ethanol production, such as whey wine distillation [46]. To overcome these challenges, alternative methods were explored. Vacuum distillation, although time-consuming, was found to preserve the natural aroma of the ethanol substrate, as well as in the spent leftover, which can be vital in certain fermentations, such as mead fermentation [47]. [48] also employed vacuum distillation in their research for ethanol dehydration and found that at higher pressures thermal decomposition occurred. However, at low pressure, such as under a vacuum, ethanol did not form an azeotrope with the components in the broth and could be theoretically separated [49].

After the ethanol distillation process, a viability test of the organisms was conducted. Viable cells were only found in the fermentation broth that underwent distillation at room temperature using a vacuum. In this work, distillation was performed to achieve the lowest possible concentration of ethanol, resulting in an ethanol-free fermented broth containing probiotic organisms used in the fermentation process. These findings suggest that distillation at room temperature using a vacuum is a suitable method for ethanol removal, as it preserves the viability of the probiotic organisms present in the fermented broth. This ethanol-free fermented broth can potentially be utilized for various applications where the presence of probiotic organisms is desired.

Probiotic tests

Tolerance tests

To further investigate the potential probiotic nature of the presumptive strains, in-vitro tolerance tests were conducted in the laboratory. The strains demonstrated viability and tolerance to bile salts, gastric juice, temperatures up to 42°C, and pH levels as low as 1.5. The details of these tests are summarized in the table 4.

Table 4. summary of in-vitro probiotic tests

Organisms	Temperature (20-42°C)	pH(6.0 -1.5)	Salt (Oxgall 5%)	Gastric juice
<i>B. megaterium</i>	+++	+	++	+
<i>B. subtilis</i>	++	+	++	+
<i>Lactobacillus sp.</i>	++	++	++	+
<i>S. cerevisiae</i>	+++	+++	++ +	+

+: Low, ++: average, and +++: good growth

Similar findings have been reported in previous studies. [22] studied the probiotic nature of *Saccharomyces cerevisiae* using similar screening methods and found that the strain could tolerate temperatures above the normal human body temperature. Grimoud *et.al.*, (2010) observed a high survival rate of *Lactobacillus* species in stimulated gastric juice and bile salt.

Stability test

Following the in-vitro tolerance testing, the viability of the presumptive probiotic strains was assessed in the whey fermented broth. The broth was treated in temperature-controlled chambers, specifically at room temperature (RT) and relative humidity (RH), at Alpha Agro R & D Laboratory. Viability tests were conducted by observing the growth of colonies on LBA and NA X-gal plates with a 10⁻⁵ times dilution. Over 10⁸ CFU/ml (colony-forming units per milliliter) cells were viable in the fermentation broth after 28 days of treatment. Coincidentally, a similar cell count of around 10⁸ CFU/ml was reported in the study by [51,52] and reviewed by [20]. To ensure the absence of cross-contamination between the presumptive probiotic cultures and any potential pathogens, a pathogenic test was conducted. This test aimed to validate the safety of the strains for probiotic applications.

Pathogenic tests

To ensure the safety of the fermented probiotic drink, a pathogenic test was conducted targeting five major

pathogens prioritized by the World Health Organization (WHO). The fermented broth that had previously passed the stability test was inoculated into specific media for each pathogen. Various indicators were analyzed according to the Indian Pharmacopeia Volume 1 guidelines. The results of the pathogenic test indicated the absence of all tested pathogens, affirming the safety of the fermented broth as a probiotic drink. A summary of the detailed pathogenic test results can be found in the **Table 5.**

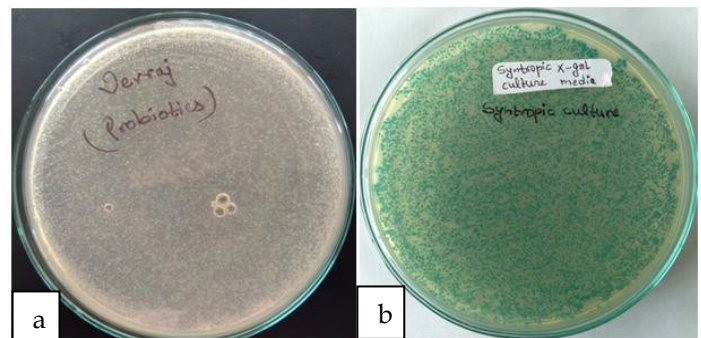


Figure 9. Colonies on a) Nutrient agar b) Nutrient agar with X-gal

These findings align with previous studies that have demonstrated the potential of probiotics in combating pathogens. For instance, a study by Howarth & Wang in 2013 investigated the effect of probiotics, specifically *Lactobacillus*, on pathogens in live chickens. The researchers observed that administering probiotics over 45 days resulted in a decrease in pathogens such as *Salmonella* and *E. coli*, while increasing the number of probiotic colonies in the chickens' fecal samples. Based on this study, it can be hypothesized that the activity of the probiotic strains or their metabolites may have contributed to inhibiting the growth of these pathogens in the whey wine during storage.

Conclusion

This study demonstrates the feasibility and potential benefits of utilizing whey to produce bioethanol and

Table 5. Summary of pathogenic test of whey wine after stability test.

S.N.	Test Pathogens	Media Used	Result/ Observation
1.	<i>Staphylococcus aureus</i>	Casein soyabean digest broth, MSA plates	Absence of yellow/ white colonies with yellow zones
2.	<i>Pseudomonas aeruginosa</i>	Casein soyabean digest broth, Cetrinide agar	Absence of greenish color colony.
3.	<i>Escherichia coli</i>	Casein soyabean digest broth, MacConkey broth and MacConkey agar	Absence of pink, non- mucoid colony
4.	<i>Salmonella</i>	Casein soyabean digest broth, Rappaport Vassiliadis Salmonella enrichment broth And Wilson and Blair's BBS agar.	Absence of uniformly black colonies surrounded by a dark zone and metallic sheen
5.	<i>Shigella</i>	Casein soyabean digest broth, GN broth and Xylose lysine deoxycholate medium.	Absence of red colored translucent colony without black center.

probiotic drinks. Whey lactose was successfully broken down into bioethanol through the co-culture of yeast and *Bacillus* Sp. Albeit with a lower ethanol concentration compared to other substrates, however, considering the abundance and renewability of whey, its utilization as a fermentation substrate offers distinct advantages over traditional food feedstock. Furthermore, the fermentation of whey resulted in a significant reduction in the organic load, indicating its potential as an environmentally friendly approach to whey management. The probiotic strains used in fermentation had tolerated different in-vitro environments making them promising candidates for probiotic applications. Stability in stimulated conditions like temperature and humidity also indicates whey as a suitable carrier for these probiotic strains. The distillation of ethanol from fermented whey provided insights into the various temperature conditions and their impact on the quality of the distillate. It was observed that distillation at room temperature using a vacuum resulted in a clear, high-quality product while preserving the inherent fragrance of the substrate. This method may have potential applications in specialized fermentation processes where maintaining specific flavors or aromas is desired.

Future research should focus on optimizing the fermentation process to enhance ethanol yields and exploring methods to increase the lactose utilization efficiency of the bacterial strains. Additionally, further investigations into the specific probiotic properties and health benefits of the isolated strains are warranted. By continuing to explore the potential of whey and its utilization in bioprocesses, we can contribute to a more sustainable and resource-efficient future.

Declaration: Ethics approval and consent to participate

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Consent for publication

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Competing interests

"The authors declare that they have no competing interests"

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