Research Article

Production of Lactic Acid from Whey Using Two Cultures Mixed Cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* and Single Cultures of *Lactobacillus plantarum*

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Abstract | This study was conducted to benefit from the lactose sugar found in whey, which is produced in large quantities by the dairy industry and poses an environmental hazard by polluting river waters, and convert it into lactic acid of high economic value in support of sustainable development using local isolates from a mixed culture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* bacteria (abbreviated as 'mixed') and a single culture of *Lactobacillus plantarum* (abbreviated as 'single')and to know the highest productivity rate between them and support with some supports and factors that help increase production, is a significant advancement in microbial fermentation research, as it allows for an accurate assessment of the effect of the organism type or cultivation conditions on biological yield. and the results indicated that the mixed culture consisting of *L. bulgaricus* and *St. thermophilus* outperformed the *L. plantarum* culture in lactic acid productivity and in all supporting factors. Yeast extract gave the highest acid production compared to other nitrogen sources, glucose was the most effective carbon source. A concentration of 0.35% of both magnesium sulfate and manganese sulfate gave the highest production, and the concentration of 15 x 10^7 of the vaccine caused the highest acid production. The pH 5.5 gave the highest acid production, while the incubation period of 48 hours was sufficient to reach the highest lactic acid production.

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Introduction

Lactic acid is one of the organic carboxylic acids; its chemical name is 2-hydroxypropionic acid, and its structural formula is $CH_3CHOHCOOH$ (Bangar *et al*., 2022). Its consumption has recently increased due to its major role in the production of many important materials, including the production of biodegradable polylactic acid, a substance with multiple applications. Currently, most lactic acid is produced through the fermentation process (de Albuquerque *et al*., 2021). It was initially believed to be one of the original components of milk, but after the discovery of fermentation processes by the scientist Louis Pasteur in 1857, it was found that it is produced by the fermentation of milk sugar by microorganisms present in it, paved the way for its commercial production through fermentation, first carried out by Avery in Littleton, USA (Oguche *et al*., 2023).

Whey is a by-product generated in large quantities during dairy manufacturing processes. It is characterized by its low pH and high chemical oxygen demand (COD). Due to the lack of effective processing technologies, whey poses an environmental challenge for the dairy industry in the context of sustainability. Recent studies across multiple disciplines have provided promising solutions to overcome this problem, such as converting it into a source of high-value compounds via biotechnology or incorporating it as a functional ingredient in food manufacturing (Luo *et al*., 2021).

The raw materials used in the production of lactic acid by fermentation on an industrial scale must have some characteristics such as low price, year-round availability, rapid fermentation, high acid production, and suitability for use without the need for pretreatment, they should produce minimal by-products during fermentation, as these increase purification costs. It is noted that most of these characteristics apply to whey (Yankov, 2022). Whey, a by-product of cheese making, poses one of the most significant challenges for dairy factory owners due to the vast quantities generated (Kaya *et al*., 2024). Global production in 2016 exceeded 100 million tons. Whey constitutes 85-95% of the volume of milk used in cheesemaking. Retains about 55% of milk's original nutrients, such as lactose, whey proteins, lipids, and mineral salts, in addition to some vitamins, an ideal medium for the production of various fermentation-derived compounds (Irazoqui *et al*., 2024).

Lactic acid can be produced by fermenting sugars by microorganisms, resulting in a pure form suitable for industrial use. This method is characterized by its low cost, and currently about 90% of this acid is produced in this way (Cheng *et al*., 2025). The fermentation process occurs via glycolysis (the Embden–Meyerhof pathway), in which a glucose molecule is converted into two lactic acid molecules, and pyruvic acid is produced as the final electron acceptor, which is reduced to lactic acid. This process is carried out by some types of lactic acid-producing microorganisms, such as *L. bulgaricus, L. helveticus*, *L. salivarius*, and *L. casei, L. delbrueckii, L. acidophilus*, and *Lactococcus lactis* (Abiola *et al*., 2022) and that some homofermenting strains such as *L. delbrueckii* and *L. helveticus* can produce more than 90% lactic acid using glucose on both laboratory and commercial scales and that genetically engineered *L. plantarum* is capable of converting pentose sugars to lactic acid through homofermentation (Chelladhurai *et al*., 2018).

Paying attention to the substrate materials used for lactic acid production is important for obtaining high yields (Tian al., 2021). It is known that carbohydrates are the main source of carbon and energy, serving as essential components for growth and metabolic activities. Yeast extract, meat extract, and peptone are excellent nitrogen sources for producing high quantities of lactic acid (Yeboah *et al*., 2023). Yeboah *et al*. (2023) said that many mineral elements are required for effective lactic acid production. The most important ones are magnesium, manganese, and iron, which are usually added as sulfate salts. Other elements that are needed include chlorine, calcium, sodium, potassium, phosphorus, and others. This is because some types of lactic acid bacteria can't grow without certain element ions, highlighting the importance of a well-balanced nutrient medium.

The aim of this study is to utilize lactose sugar present in whey, which is produced in large quantities in the dairy industry and is not utilized, and convert it into lactic acid of high economic value using local isolates from a mixed culture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* bacteria and a single culture of *Lactobacillus plantarum* and to know the highest productivity rate among them.

Materials and Methods

A mixed culture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* was isolated from yogurt and morphologically identified. A singl culture of *Lactobacillus plantarum* was also obtained and laboratory identified in the Food Science Laboratories at the University of Mosul.

Whey was obtained as a by-product of white cheese manufacturing from buffalo milk by an enzymatic method using microbial rennet. It was then heated at

90°C for 20 min to precipitate proteins, which were removed by centrifugation at 4000 × g for 15 min following the procedure described by Mawgoud *et al.* (2016).

Estimation of lactic acid

The method was followed by Borshchevskaya *et al.* (2016) with some modifications. The method is based on measuring the light absorption of the yellow-green ferric lactate complex produced by the reaction of lactate with ferric chloride ($FeCl_3$) at a wavelength of 405 nm using a spectrophotometer manufactured by APEL, Japan. Lactic acid detection was carried out using the computational method mentioned by (Ghaly *et al.*, 2003) and according to the law:

$$Production\ rate\ (g/L/h) = \frac{Product\ quantity}{Fermentation\ time\ (hour)}$$

The effect of some factors on the production of lactic acid from whey was studied by the meal method and using the mixed culture of *S. thermophilus*, *L. bulgaricus* and *L. plantarum* incubated at 40°C. The method of Chavarri *et al.* (2010) was followed in preparing the vaccine of isolates This process was carried out by centrifuging a quantity of bacterial culture grown in liquid medium at 5000×g for 15 minutes with cooling. The precipitate was collected and washed with peptone water, then the centrifugation process was repeated. The precipitate was suspended in 10 ml of peptone water. The count of the two types of bacteria was carried out by the pour plate method using MRS agar medium., including the type and concentration of carbon and nitrogen sources, some mineral elements, concentration and age of the sample, in addition to the effect of the acidity function and the duration of incubation.

Effect of nitrogen source type on lactic acid production

Some nitrogen sources were added at a concentration of 10 g/L to the whey medium used in acid production. The added sources included yeast extract, urea, peptone, meat extract, sodium nitrate, ammonium nitrate, ammonium chloride, and ammonium nitrate. After fermentation, each of the lactic acids was estimated. As for the effect of yeast extract concentration on lactic acid production, concentrations of 2-20 g/L were used, after which the fermentation process was carried out, and then the amount of lactic acid was estimated.

Effect of carbon source type on lactic acid production

The whey medium containing the optimum concentration of yeast extract was supplemented with some sugars as carbon sources at a concentration of 10 g/L. The fermentation and acid production process was carried out, and after the fermentation was completed, the amount of lactic acid was estimated.

The effect of added lactose concentration on lactic acid production

The whey medium containing the optimal concentration of yeast extract was supplemented with lactose sugar at concentrations ranging from 5 to 50 g/L, and a process was carried out, and then the amount of lactic acid produced was estimated.

Effect of magnesium sulfate concentration on lactic acid production

Magnesium sulfate was added, at concentrations ranging from 0.05 to 0.5 g/L, to the whey medium supplemented with the optimal concentrations of yeast extract and lactose. Then, the fermentation process was carried out using a mixture of bacteria and lactic acid that was estimated.

Effect of manganese sulfate concentration on lactic acid production

Manganese sulfate was added, at concentrations ranging from 0.01 to 0.1 g/L, to the whey medium supplemented with the optimal concentrations of yeast extract and lactose. The fermentation process was carried out using a mixture of bacteria and lactic acid that was estimated.

Effect of vaccine concentration on lactic acid production

The bacterial mixture vaccine, at concentrations ranging from 5-20 × 107 cells/ml, was added to the whey medium supplemented with the optimal concentrations of yeast extract, lactose, magnesium sulfate, and manganese sulfate. Then, the fermentation process was carried out, and lactic acid was determined.

Effect of pH on lactic acid production

After preparing the whey medium supplemented with the above-mentioned materials, the pH was adjusted to values ranging from 4 to 8 by hydrochloric acid or sodium hydroxide (1 molar), then the medium was sterilized, the vaccine was added, and the fermentation process was carried out. After the fermentation period was over, lactic acid was estimated.

Effect of incubation period on lactic acid production

The incubation process of the whey medium inoculated with the bacterial mixture was carried out for periods ranging from 6 to 48 hours. The amount of lactic acid produced was estimated at the end of the incubation periods.

Statistical analysis

The data were analyzed using a simple experiment with a completely randomized design (CRD) to identify the nature of differences between treatment levels. Duncan's multiple range test was used to determine the significance of differences between means at a probability level of 0.01. The SAS statistical software was used, and the statistical analysis was conducted based on three replicates for each treatment.

Results and Discussion

Effect of culture type on production quantity

Figure 1 shows that there is a significant difference between the lactic acid production rate of the mixture of two types of cultures and single cultures from whey, as the mixture of bacteria gave a production rate of 15.92 g/L, which is higher than the production of single cultures bacteria, which reached 7.68 g/L, respectively. Mixed cultures offer metabolic synergy compared to single cultures, as each species complements the other's functions in terms of better utilization of sugars, higher acid tolerance, greater efficiency in converting to lactic acid, and longer production continuity, which led to the acceleration of acid production, while the reason for the decrease in acid production in single cultures was that it produces external enzymes other than lactase.

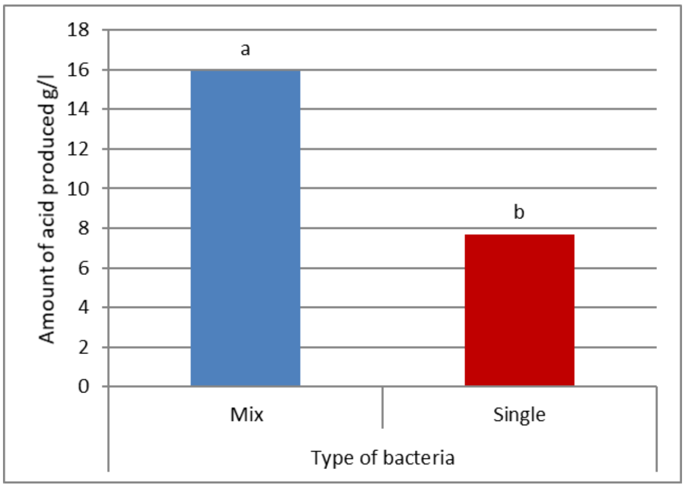

Figure 1: *The difference between the production of lactic acid by mixed and single bacteria.*
*Mix : L. bulgaricus and Str. thermophilus, Single : L. plantarum. *Values are the average of three replicates. *Letters above columns indicate significant differences at the 0.01 level.*

Effect of nitrogen sources on production

Figure 2 indicates that there are significant differences between the productivity of lactic acid by a mixture of two types of bacteria and single cultures bacteria from whey supplemented with a number of nitrogen sources, namely yeast extract, peptone, urea, Cl_4NH, and $(NH_4)_2SO_4$. The mixture of cultures outperformed L. plantarum in acid production in all treatment sources. Yeast extract gave the highest acid productivity, reaching 2.61 and 1.52 g/L for each of Mix and single cultures, respectively, while $(NH_4)_2SO_4$ and Cl_4NH did not give any differences in increasing productivity because they are inorganic nitrogen sources. Because yeast extract provides a rapidly absorbable source of nitrogen compared to other nitrogen sources, which enhances the metabolic activity of bacteria. The lowest productivity was when using $(NH_4)_2SO_4$, reaching 1.50 and 0.83 g/L for each of Mix and single cultures, respectively. Taleghani *et al.* (2016) reached a similar conclusion, noting that yeast extract was superior to both meat extract and peptone in increasing acid production from *L. bulgaricus* grown in whey. Abdel-Rahman *et al.* (2019) concluded that the best nitrogen sources for Enterococcus hirae growth and acid production in MRS medium were a mixture of yeast extract, peptone, and meat extract, which gave a 22% increase in production compared to the medium without them. Hakobyan *et al.* (2012) reported that yeast extract was better than free amino acids as a nitrogen source for bacterial growth because it contains a complete set of amino acids and vitamins, especially vitamin B complex, in addition to other growth-stimulating compounds and elements.

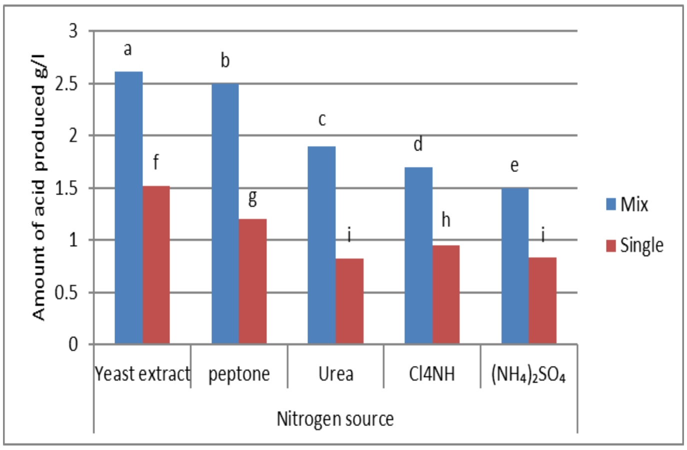

Figure 2: *The effect of adding nitrogen sources on lactic acid production.*
*Mix : L. bulgaricus and Str. thermophilus, Single : L. plantarum. *Values are the average of three replicates. *Letters above columns indicate significant differences at the 0.01 level.*

The effect of carbon sources on production

Figure 3 shows significant differences between the productivity of lactic acid by a mixture of two types of cultures and single cultures bacteria from whey supplemented with a number of carbon sources, namely lactose, glucose, fructose, sucrose, and galactose. The mixture of cultures outperformed single in acid production in all treatment sources, and glucose sugar outperformed the rest of the sugars in increasing productivity, while galactose gave the lowest acid productivity. The highest lactic acid productivity was in the presence of glucose, reaching 35.5 and 20.3 g/L for both Mix and single, respectively, and the lowest lactic acid productivity was in the presence of galactose, reaching 25.9 and 11.3 for both Mix and L. plantarum, respectively. Because glucose enters directly into the glycolysis pathway, which allows it to be converted directly into pyruvic acid and then into lactic acid without the need for additional steps or enzymes. Another, this is what Sobowale *et al.* (2011) reached to a similar result when they studied the effect of some carbon sources on the growth of lactic starter bacteria (*S. thermophilus* and *L. bulgaricus*), as they found that glucose and lactose were better than other sugars in stimulating growth, and growth was weak when using galactose alone, and growth improved when using a mixture of galactose and sucrose.

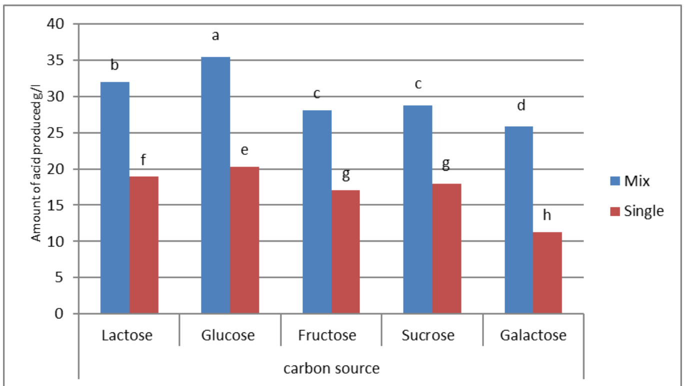

Figure 3: *The effect of adding carbon sources on lactic acid production. Mix : L. bulgaricus and Str. thermophilus, Single : L. plantarum. *Values are the average of three replicates. *Letters above columns indicate significant differences at the 0.01 level.*

Effect of magnesium sulfate on production

Figure 4 shows that adding multiple concentrations of magnesium sulfate as a type of mineral fortification to the whey inoculated with a mixture of two types of cultures and single cultures caused an increase in lactic acid production. Acid production increased as the concentration of magnesium sulfate added increased until it reached a concentration of 0.3, which had no significant differences with a concentration of 0.4. The mixture cultures outperformed single cultures in acid production at all concentrations used. The highest amounts of acid production were at a concentration of 0.4 and reached 39.5 g/L for Mix, while the highest amount of acid production by single cultures at a concentration of 0.3 reached 27.3 g/L. The reason for the increase in lactic acid production when magnesium sulfate is added is that it acts as a cofactor for vital enzymes, which leads to increased bioconversion efficiency. The results agreed with Bouhadi *et al.* (2017) when growing S. thermophilus bacteria in a molasses medium, as adding magnesium sulfate to the whey medium at a concentration of 0.5 g/L to get 46.26 g/L of lactic acid.

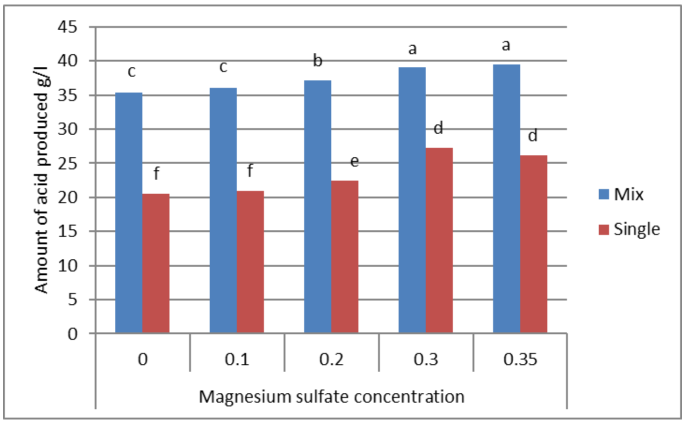

Figure 4: *The effect of adding magnesium sulfate on lactic acid production. Mix : L. bulgaricus and Str. thermophilus, Single : L. plantarum. *Values are the average of three replicates. *Letters above columns indicate significant differences at the 0.01 level.*

Effect of manganese sulfate on production

Figure 5 shows that adding multiple concentrations of manganese sulfate as a type of mineral fortification to the whey inoculated with a mixture of two types of cultures and single cultures caused an increase in lactic acid production. Acid production increased as the concentration of manganese sulfate added increased until it reached a concentration of 0.3, which had no significant differences with a concentration of 0.4. The mixture of cultures outperformed single cultures acid production at all concentrations used. The highest amounts of acid production were at a concentration of 0.4, reaching 41.80 and 23.51 g/L for each of Mix and single, respectively. Adding manganese sulfate enables bacteria to withstand difficult fermentations such as increased acidity resulting from the accumulation of lactic acid, which prolongs the production period. This is what some researchers indicated: adding manganese sulfate to the whey medium containing *L. bulgaricus* bacteria increased lactic acid production by 23.3% compared to the medium without it (Mawgoud *et al.*, 2016).

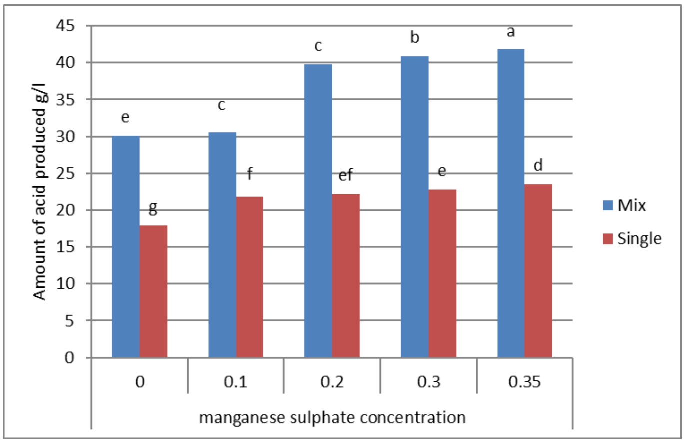

Figure 5: *The effect of adding manganese sulfate on lactic acid production.*
*Mix : L. bulgaricus and Str. thermophilus, Single : L. plantarum. *Values are the average of three replicates. *Letters above columns indicate significant differences at the 0.01 level.*

Effect of vaccine concentration on production

Figure 6 shows the direct increase in the amount of lactic acid with increasing vaccine concentration, as the vaccine was added at three concentrations to the whey medium: 5, 10, and 15 x 10^7 (cells/ml) with few significant differences, and the concentration 15 x 10^7 gave the highest production of acid, reaching 41 (g/L) for the mixed culture, and single cultures gave (30.11 g/L) at the same vaccine concentration. The results were close to what was mentioned by Minh (2014) when using the concentration of 9.4 x 10^9 cells/ml of single cultures vaccine in the production of lactic acid from corn grain waste extract.

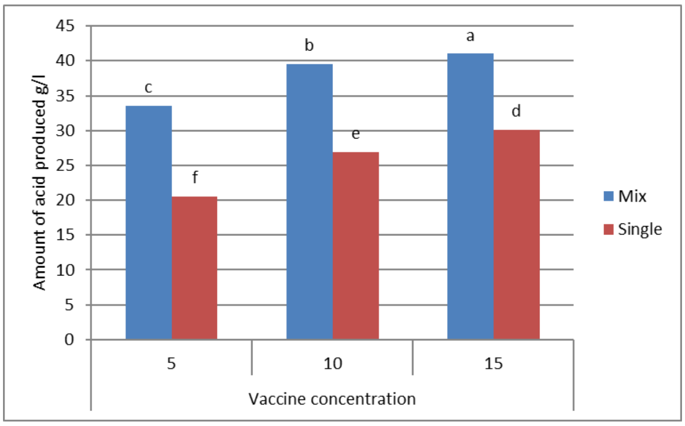

Figure 6: *Effect of specific concentrations of vaccine on lactic acid production.*
*Mix: L. bulgaricus and Str. thermophilus, Single : L. plantarum. *Values are the average of three replicates. *Letters above columns indicate significant differences at the 0.01 level.*

Effect of pH on production

Figure 7 shows the use of pH values between 4-8, and the results showed that the production quantity reached its maximum value at (5.5) pH for the pH of the whey medium for both cultures. At this pH, the main enzymes in the lactic acid production pathway operate at their highest efficiency, accelerating the conversion of pyruvate to lactic acid. with a significant and clear difference between the beginning and end of the addition, as production reached (43.1 g/L) for the mixed culture and (21.93 g/L) for the single cultures. The pH has a significant effect on the effectiveness of various cellular enzymes and on the transport of nutrients through the cell wall, as well as on the degree of solubility and ionization of cellular compounds, and thus on the cell's metabolism and production of various compounds (Joshi and Amrutsagar 2017).

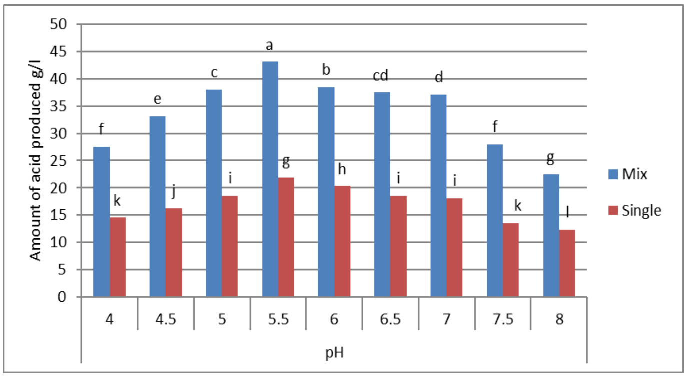

Figure 7: *Effect of pH on lactic acid production.*
*Mix: L. bulgaricus and Str. thermophilus, Single : L. plantarum. *Values are the average of three replicates. *Letters above columns indicate significant differences at the 0.01 level.*

The effect of incubation duration on production

Figure 8 shows the incubation periods, which ranged between 6 and 48 hours. It is noted that the productivity increased significantly, reaching the maximum after 48 hours of inoculation, as the produced quantity reached 40.5 g/L for the mixed culture and 36.3 g/L for the single cultures. The result obtained was close to that reached by Luongo *et al.* (2019), as they reached the maximum production of acid (20.1 g/L) after 48 hours of incubation in the whey medium and with a semi-continuous process of feeding at regular intervals with the product withdrawn at the same rate. The reason is due to bacteria reaching a stationary phase where growth stops due to a lack of nutrients or the accumulation of toxic products (such as the acid itself). Here, bacteria continue to produce acid at high efficiency even as growth slows. The effect of symbiosis is that *S. thermophilus* produces formic acid, which stimulates the growth of *L. bulgaricus*. *L. bulgaricus* provides amino acids such as valine and histidine, which support the growth of S. thermophilus. The high lactic acid production peaks within 48 hours due to this synergy, while the single culture may take longer (up to 72 hours) to reach peak production compared to mixed cultures.

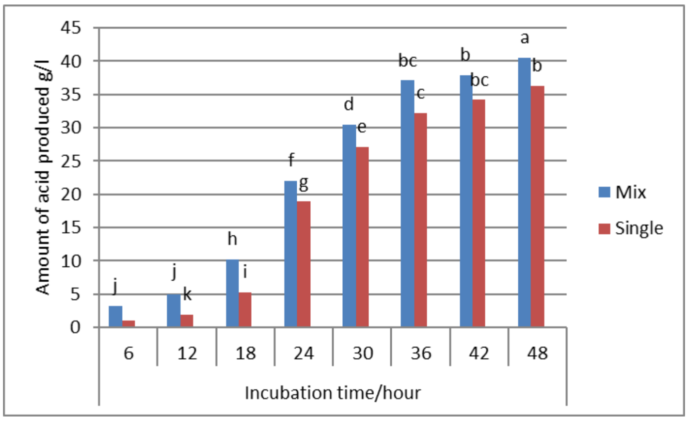

Figure 8: *Effect of incubation period on lactic acid production. Mix: L. bulgaricus and Str. thermophilus, Single: L. plantarum. *Values are the average of three replicates. *Letters above columns indicate significant differences at the 0.01 level.*

Conclusions

These results showed that the mixture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* cultures produced a higher yield of lactic acid compared to *Lactobacillus plantarum* cultures, and that the supporting materials and factors that help increase production did not alter this outcome that the mixture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* cultures was more productive than *Lactobacillus plantarum* cultures. The results showed that the bacterial mixture (*L. bulgaricus* + *S. thermophilus*) is superior in lactic acid production compared to *L. plantarum*. Can be attributed to three main metabolic advantages: The symbiotic effect between the two strains, where S. thermophilus stimulates the growth of *L. bulgaricus* by producing formic acid and amino acids, while *L. bulgaricus* provides protease enzymes to break down proteins; efficient sugar conversion, as the mixture has integrated metabolic pathways that ferment lactose more efficiently; and acidity tolerance, as the synergy between them delays enzyme inhibition at low pH, compared to *L. plantarum*, which works alone. This study contributed to future directions for improving lactic acid production from whey: Scaling up the pilot scale by testing optimum conditions on an industrial scale, improving through genetic engineering by modifying *Lactobacillus* and *Streptococcus* strains to increase acidity tolerance, enhancing metabolic pathways for lactic acid production using genetic modification tools, and developing continuous fermentation systems.

Novelty Statement

This research pioneers an efficient lactic acid production method by uniquely comparing mixed cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* with single cultures of *Lactobacillus plantarum*, specifically utilizing whey as a sustainable substrate. This comparative study offers novel insights into optimizing fermentation strategies for enhanced lactic acid yield and offers a sustainable approach to valorizing dairy by-products

Author's Contribution

Sahar Adnan Sheet: Formulating the idea and general supervision, theoretically and practically
Ruaa Adel Hamed: Help manage the article and conduct practical experiments.
Zaman Nazem Taher: Provided technical input.
Anas Saad Hatem: Helped in management of article.

Conflict of interest

The authors have declared no conflict of interest.

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