Determining Optimal Conditions for Lactobacillus Paracasei Bacteria and Testing its Ability to Produce Conjugated Linoleic Acid

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Abstract: The study included testing the ability of some bacteria to produce conjugated linoleic acid (CLA) and identifying the most efficient isolates for production. The results showed that seven species of the Lactobacillus genus were isolated, namely (Lactobacillus paracasei, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus helveticus, Lactobacillus rhamnosus, Lactobacillus reuteri). The results indicated that all bacterial isolates had the capability to produce CLA when cultivated in MRS Agar medium. Each isolate was subjected to a screening process to determine the most efficient in producing CLA under various conditions. The results showed that the isolate Lactobacillus paracasei was the most efficient in producing CLA. Additionally, the optimal conditions for producing CLA by Lactobacillus paracasei were determined, and its ability to produce CLA under different conditions was tested. The results indicated that the optimal pH for production by Lactobacillus paracasei was 6, and the optimal temperature for producing CLA was 37°C. The incubation period for producing CLA showed that 24 hours was the best duration for fatty acid production. A concentration of 250 µg/ml of CLA resulted in the highest yield of the conjugated acid for all isolates, with the isolate Lactobacillus paracasei showing superior production of CLA.

1 INTRODUCTION

Conjugated linoleic acid (CLA) is a polyunsaturated fatty acid (PUFA) from the w-6 series, consisting of a variety of geometric and positional isomers (cis or trans) of linoleic acid (LA; cis-9,12-octadecadienoic acid, 18:2) with a conjugated double bond, and has garnered increasing attention in recent decades due to the discovery of many health benefits associated with it [1], [2].

CLA is found in various natural foods, such as vegetable oils like safflower oil, sunflower oil, corn oil, soybean oil, and sesame oil. It is also present in the meat and milk of ruminant animals [3].

Researchers, after identifying the ability of certain lactic acid bacteria and probiotics to secrete the enzyme Linoleic Acid Isomerase, have turned to producing CLA outside the living organism using microorganisms with various nutrient media supplemented with linoleic acid, as well as alternative sources of pure linoleic acid, such as safflower oil, sunflower oil, soybean oil, and others due to their high content of this fatty acid. Following the success achieved in producing CLA outside the living organism and from alternative sources rather than pure acid, a significant step has begun in incorporating these techniques into foods, including dairy products and cheese, to create dietary systems that serve as a benchmark against other diets due to their content of probiotics, in addition to CLA and the benefits provided by the product [4].

2 MATERIALS AND METHODS

Bacteria (Lactobacillus paracasei, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus helveticus, Lactobacillus rhamnosus, Lactobacillus reuteri) were obtained from laboratories affiliated with the Holy Hussain Shrine in Karbala, packaged in sterile, airtight containers.

2.1 Activation of Bacterial Isolation

1 g of samples was taken, and a series of tenfold dilutions 10 - 10 - 7 was performed in peptone water. 1 ml of it was transferred to the culture medium MRS Agar, and the plates were incubated under anaerobic conditions using an Anaerobic jar at 37°C for 48 hours, following the method used by [5].

2.2 Test for the Ability of the Studied Bacteria to Produce Linoleic Acid

The screening of the initially identified isolates was conducted based on their ability to convert linoleic acid into CLA, according to the method described by [6]. As follows: Bacterial isolates were activated in MRS Broth medium containing 1% Tween 80 (w/v) and 1 µg/ml of pure linoleic acid, and the tubes were incubated at 37°C for 24 hours.

2.3 The Standard Curve for CLA

It was prepared according to the method followed by [8]. Different concentrations of the standard CLA (Cis9 Trans11) ranging from 0 to 12 micrograms/mL dissolved in hexane, were made, and the absorption was measured at a wavelength of 233 nanometers (Fig. 1).

2.4 Calculation of the Conversion Ratio of Linoleic Acid to CLA

The percentage of conversion capability was calculated according to the method followed by [7]. From the following (1):

$$\text{CLA acid \%} = \frac{\text{Amount of CLA obtained from the standard curve}}{\text{Amount of added linoleic acid (ml or g) standard}} \quad (1)$$

2.5 Determining the Optimal Conditions for the Production of CLA

In the broth medium, CLA optimal conditions were determined to test the bacteria's ability to produce CLA. 10 mL of the growth medium 10 microliters of 80 Tween, MRS microliters from safflower oil, and 500 microliters of the extracted bacteria were used and incubated in the shaker device for 4 days [9]:

- The effect of pH. The used ranges of PH are (4,5,6 and 7).
- Effect of temperature. For culturing liquid MRS medium, the temperatures (20,30,37,40°C) were used contained linoleic acid 1 µg/ml and inoculated with 2% of the active cultures to determine the optimal temperature.
- Effect of Safflower Oil Concentration. Different concentrations of safflower oil (50,100,200,300,400) µ /ml were used.
- Effect of incubation duration. Different incubation periods (6,12,24,48,72)hours were applied.

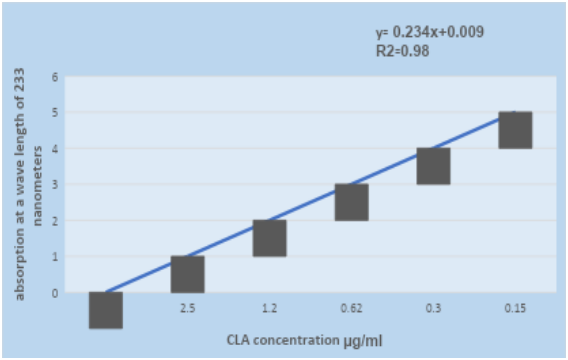

Figure 1: The standard curve for the measured CLA.

3 RESULTS AND DISCUSSION

3.1 Quantitative Screening to Identify Isolates with Highest Production of CLA

Many researchers have used spectroscopic methods for the detection and quantification of CLA due to their numerous advantages. They do not require the esterification process as in chromatography, have a low cost, and allow for the screening of a large number of samples. Furthermore, some researchers have used this method [12] and [8].

A screening process was conducted on the studied bacteria to evaluate their ability to produce CLA and the quantity produced. Table 1 illustrates the variation in the isolates' ability to produce CLA. The highest concentration of CLA reached 113.15 µg/L for the isolate Lactobacillus paracasei, followed by the isolates Lactobacillus casei, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus helveticus, Lactobacillus rhamnosus, and Lactobacillus reuteri, with concentrations of 98.45, 79.11, 77.48, 69.93, 57.12, and 53.56 µg/L, respectively. The conversion percentages were

11.31%, 9.84%, 7.91%, 7.74%, 6.99%, 5.71%, and 5.35%, respectively.

The achievement of the highest production of CLA from the isolate Lactobacillus paracasei under static anaerobic conditions may be attributed to the microaerophilic nature of this isolate. Furthermore, the static culture flask provides an almost unlimited number of variable fermentation conditions, ranging from nutrient enrichment to depletion, and from abundant oxygen supply to partial anaerobic coexistence.

A study has shown that the process of screening and selecting the best organisms for the production of CLA revealed that Lactobacillus bacterial strains exhibit a production range of CLA between 20 and 4900 µg/L [15].

Table 1: This caption has one line so it is centered.

Bacterial isolation	Quantity of CLA product µg/L	CLA% percentage
Lactobacillus paracasei	113.15	11.31
Lactobacillus casei	98.45	9.84
Lactobacillus acidophilus	70.11	7.91
Lactobacillus plantarum	77.48	7.74
Lactobacillus helvticum	69.93	6.99
Lactobacillus rahmanosus	57.12	5.71
Lactobacillus reuteri	53.56	5.35

In a study conducted by [7], the results of isolating Lactobacillus rahmanosus showed the lowest production of CLA, amounting to 46.2 µg/L, with a conversion rate of 4.62%. On the other hand, [16] indicated the ability of lactic acid bacteria to produce CLA in skim milk medium prepared with linoleic acid at a concentration of 200 µg/mL. The bacterial isolate Lactobacillus acidophilus 6 gave the highest production, reaching 116.53 µg/mL, with a conversion rate of 58.26%, while the bacterial isolate Lactobacillus casei had the lowest CLA production, amounting to 71.36 µg/mL, with a conversion rate of 35.68%. The study also pointed out that using concentrations higher than 200 µg/mL resulted in a decrease in the amount of CLA, attributing this to the inhibitory effect of linoleic acid on the bacterial isolates.

3.2 Determination of the Optimal Conditions for the Production of CLA in the Reaction Medium.

3.2.1 The Effect of pH

The results in Figure 2 showed that when using pH values (4, 5, 6, 7) in the production of CLA from the L. paracasei producing isolate, the optimal pH for CLA production was 6, where the amount of linoleic acid reached 168.11 µg/L with a conversion rate of 16.2% for the producing isolate.

The results shown in Figure 2 when using pH values (4, 5, 6, 7) in the production of CLA from the producing isolate L. paracasei indicated that the optimal pH for the production of CLA is 6, as the amount of linoleic acid reached 168.11 µg/L, with a conversion rate of 16.2% for the producing isolate."

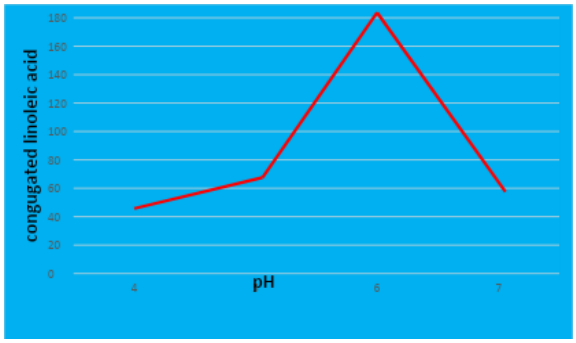

Figure 2: Effect of different pH levels on the production of CLA.

The pH of the medium is crucial for the growth of living organisms and its impact on metabolism. Changes in pH are significant for the effectiveness of enzymes in living organisms, as well as for intermediate products, their solubility, and decomposition. These changes have an effect on the yield of metabolic products of living organisms.

pH affects metabolic enzyme function and nutrient transport in the cell [10]. In a study conducted [18], at pH 6, in the production of CLA, it was possible to produce it from L. acidophilus bacteria, which achieved the best growth and the best production of CLA.

Maintaining a narrow pH range is essential for optimizing productivity in culture media. pH regulation is a critical factor, and therefore, compounds that act as pH buffers are added to culture media to maintain the desired pH. These buffers also serve as nutrient sources for microorganisms. Calcium carbonate is commonly used to maintain a neutral pH in the medium. When

the pH decreases, the carbonate dissolves, neutralizing the acidity. Conversely, when the pH increases, acids released into the medium by microorganisms help to lower the pH back towards neutrality (pH 7).

3.2.2 Temperature Effect

The results in Figure 3 showed that the highest production of CLA for the L.paracasei isolate when using different temperatures (20, 30, 37, 40 °C), was at 37 °C, where the CLA production reached 161.47 µg/L, with a conversion rate of 16.1%. Compared to the other temperatures, the amount of CLA production was (112.73, 97.47 µg/L) at 20 and 40 °C, respectively.

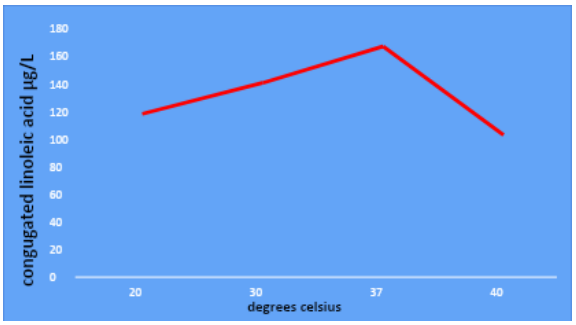

Figure 3: Effect of different temperatures on CLA production.

The increase in CLA productivity with rising temperature may be attributed to making the reaction medium conducive to the growth of microorganisms and the secretion of isomerase enzyme without causing any effect or change in the enzyme's function, while providing the substrate on which the enzyme acts. However, the decrease in CLA productivity when incubated at 40°C can be attributed to the effect on enzyme activity due to thermal inhibition of the enzyme [19]. These results are consistent with those obtained by [11] in his study, where he found that the optimal incubation temperature is 37°C, and that an increase in incubation temperature caused a sharp decrease in conjugated acid productivity. As a result, the increase in temperature affects hydrogen migration during the formation of isomers, and stopping enzyme activity causes hydrogen migration to stop completely. These results also agree with what [8] found, that the optimal incubation temperature ranged between 35 and 37°C, and that an increase above this temperature caused a sharp decrease in the production of conjugated acid, explaining the reason as the enzyme being sensitive to temperature, and that the increase in incubation temperature caused the enzyme structure to be destroyed. On the other hand, a study [16] showed that the best incubation temperature for producing CLA in MRS broth medium was at 37°C, and confirmed a sharp decrease in the production of conjugated acid upon reaching 40°C, explaining the reason for this decrease as a result of the decrease in enzyme activity.

3.2.3 The Effect of Linoleic Acid Concentration

The concentration of linoleic acid is considered one of the key factors that significantly influence the production of CLA in the reaction medium [8]. Due to its importance, it was studied under optimal conditions to determine the best production of CLA. Figure 4 illustrates the effect of different concentrations of linoleic acid on the production of CLA in MRS broth supplemented with concentrations of (50, 100, 200, 300, 400) µg/mL and inoculated with the local bacterial isolate, Lactobacillus paracasei. It was observed from the same figure that there was a gradual decrease in the concentration of CLA with the increase in the concentration of linoleic acid added to the reaction medium. The concentration of 50 µg/mL outperformed, reaching (181.15) µg/mL with a conversion rate of 18.1%. The concentrations of (100, 200, 300, 400) µg/mL resulted in (171.66, 164.11, 158.13, 36.22) µg/mL, respectively, in CLA production. The reason for the increase in CLA production at low concentrations may be attributed to the unaffected structural conformation of the isomerase enzyme secreted by the microorganisms, which is responsible for CLA production, thus increasing the surface area for enzyme binding with the substrate in the reaction medium. The decrease in CLA production in the reaction medium may be due to the gradual decrease in bacterial counts, as well as the effect of increased fatty acid concentration on the secretion of the isomerase enzyme, or due to the reduction in the surface area for enzyme binding with its substrate, leading to a decrease in acid productivity. This is supported by [8], who found that the concentration of CLA was significantly affected by microbial cell concentrations and linoleic acid concentration in the reaction medium, and that high concentrations of the substrate cause changes in the enzyme structure, as well as reducing the surface area for enzyme binding with its substrate in the reaction medium.

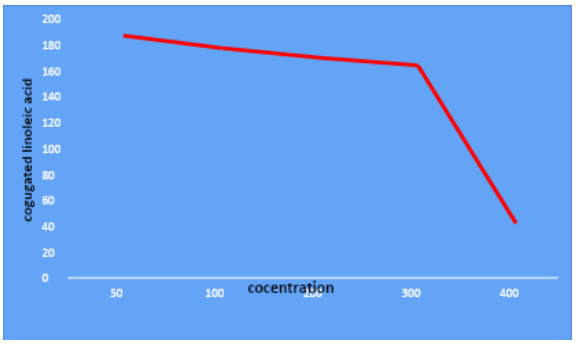

Figure 4: Effect of different concentrations on production.

3.2.4 Effect of Incubation Period

Figure 5 illustrates the effect of different incubation periods, ranging from (12, 24, 48, 72) hours, on the ability of the selected bacterial isolate L. paracasei to produce CLA using MRS broth, incubated at 37°C. The results for the different periods were (0, 14.92, 16.157, 32.140, 76.122) µg/mL, respectively. The results showed that at the initial incubation period, no CLA was formed, accompanied by a decrease in microbial counts. This is attributed to the microorganisms entering a lag phase to prepare the necessary enzymes for their vital activities.

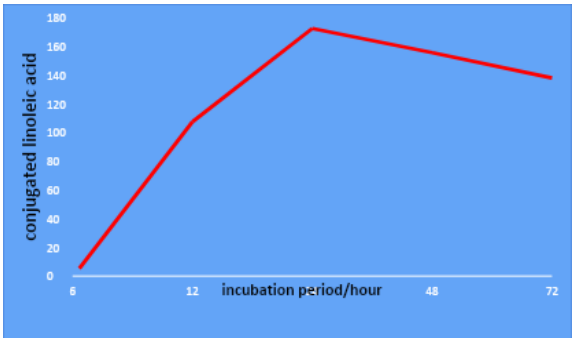

Figure 5: Effect of incubation periods on CLA production.

During this period, a decrease in microbial counts may occur due to the effect of linoleic acid in the production medium, as it is one of the factors that hinder microbial growth. The highest production of CLA was achieved at 24 hours, with a conversion rate of 27.15%. These results are consistent with several researchers who indicated that the optimal time for CLA production is at the end of the logarithmic phase and the beginning of the stationary phase, and they showed that the optimal incubation period for CLA production is 24 hours [13], [16]. In a study conducted by [6], [21], the optimal incubation period using MRS broth supplemented with 1 µg of CLA was 24 hours. The decrease in the amount of CLA with prolonged incubation periods is due to oxidation reactions, as well as oxidation resulting from microbial metabolism. During his study [9], found that the optimal incubation period is 48 hours. This was confirmed by [13], who found that the optimal incubation period for producing CLA was 48 hours. He also stated that the optimal period for producing CLA was during the stationary phase.

4 CONCLUSIONS

The current study demonstrates that Lactobacillus bacteria, in general, have the ability to produce linoleic acid when cultured on various growth media. Changes in pH, incubation time, or concentration can result in the production of varying amounts of the acid. This study successfully demonstrated the potential of various *Lactobacillus* species to biosynthesize CLA from linoleic acid in vitro, with *Lactobacillus paracasei* emerging as the most efficient producer. The isolate produced the highest concentration of CLA (113.15 µg/L) with a conversion rate of 11.31%, outperforming other species under standardized conditions.

These findings highlight the critical role of environmental conditions in modulating microbial CLA biosynthesis, primarily due to their influence on enzyme activity and microbial metabolism. Elevated linoleic acid concentrations beyond the optimal threshold were found to inhibit CLA production, likely due to enzyme feedback inhibition or toxicity.

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