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Original Article

Partial purification of linoleic acid isomerase enzyme from *Lactobacillus paracasei* bacteria isolated from milk

Purificação parcial da enzima isomerase do ácido linoleico da bactéria *Lactobacillus paracasei* isolada do leite

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Abstract

Conjugated Linoleic Acid (CLA) has attracted the attention of many researchers, especially that of microbial origin due to its biological importance to the consumer. The current study aims to extract LA Isomerase enzyme from *Lactobacillus paracasei* bacteria from milk and to use the enzyme in the production of CLA. Selective media, including MRS and MRS-Dagatose, were used in isolating local strains. The selected bacterial isolates were tested for their ability to produce LA-Isomerase enzyme. The isolate with high enzymatic activity was selected. After extraction and partial purification of the enzyme, the optimal conditions for the production of conjugated fatty acid were studied, and the reaction products were diagnosed using GC-MS technology. It was found that 11 isolates have the ability to produce CLA at different concentrations, H_1 isolate showed the highest production of conjugated fatty acid at a concentration of 120.45 g.ml^{-1}, this isolate was selected as the source for enzyme extraction. The enzymatic activity of the crude extract and partially purified with ammonium sulfate was estimated using color methods at wavelength of 233 nm. The effect of the optimum conditions (pH, temperature, linoleic acid concentration and enzyme concentration) on the CLA product was studied using the partially purified LA Isomerase enzyme, the optimum conditions for production were 6.5, 45 °C, 100 µg.ml^{-1} and 0.7 ml, respectively. The GC-MS technique showed the presence of a number of reaction products that are isomers of conjugated linoleic acid (C_9T_{11}, T_9T_{12}, $T_{10}C_{12}$) with different concentrations.

Keywords: lactobacillus, CLA, linoleic acid, LA isomerase, human milk.

Resumo

O Ácido Linoleico Conjugado (CLA) tem chamado a atenção de diversos pesquisadores, principalmente aquele de origem microbiana, devido à sua importância biológica para o consumidor. O presente estudo visa extrair a enzima LA Isomerase da bactéria Lactobacillus paracasei do leite e usar essa enzima na produção de CLA. Meios seletivos, incluindo MRS e MRS-Dagatose, foram usados no isolamento de cepas locais. Os isolados bacterianos selecionados foram testados quanto à sua capacidade de produzir a enzima LA-Isomerase. Foi selecionado o isolado com alta atividade enzimática. Após a extração e purificação parcial da enzima, as condições ideais para a produção de ácido graxo conjugado foram estudadas e os produtos da reação foram identificados usando a tecnologia GC-MS. Verificou-se que 11 isolados possuem capacidade de produzir CLA em diferentes concentrações. O isolado H1 apresentou a maior produção de ácido graxo conjugado, na concentração de 120,45 g.ml^{-1}, e este isolado foi selecionado como fonte para extração enzimática. A atividade enzimática do extrato bruto e parcialmente purificado com sulfato de amônio foi estimada por métodos de coloração em comprimento de onda de 233 nm. O efeito das condições ótimas (pH, temperatura, concentração de ácido linoleico e concentração de enzima) no produto CLA foi estudado usando a enzima LA Isomerase parcialmente purificada e as condições ótimas para produção foram 6,5, 45 °C, 100 µg.ml^{-1} e 0,7 mL, respectivamente. A técnica de GC-MS mostrou a presença de uma série de produtos de reação que são isômeros do ácido linoleico conjugado (C9T11, T9T12, T10C12) com diferentes concentrações.

Palavras-chave: lactobacillus, CLA, ácido linoleico, LA isomerase, leite humano.

1. Introduction'

Human milk is an elixir for newborns, providing a wealth of nutrients and beneficial microbiota that are essential for infant growth and development. Its advantages prompted studies into the components of milk and their potential use as prophylactic or therapeutic agents. Although culture-independent milk microbiome estimation

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and high-resolution milk component identification provide data, there is a lack of a comprehensive view of these research domains (Souza et al., 2016; Lima et al., 2018). Milk is a complex biological liquid that meets the nutritional requirements of infants, as it works to develop the immune system and protect the child from diseases through its biologically active ingredients in both colostrum and milk (George et al., 2018; Ray et al., 2019; Auer et al., 2021). A number of studies have proven that colostrum and milk are a rich source of many important microorganisms that are transferred to infants during the breastfeeding process, and it is the main source of beneficial bacteria in the digestive system (Wheeler et al., 2007; Toscano et al., 2017; Niyazbekova et al., 2020; Fasse et al., 2021). It has been observed that breast milk is supplied with approximately 10^5-10^7 beneficial bacteria during one feeding (Martín et al., 2009).

Milk is a good source of unsaturated fatty acids, especially conjugated linoleic acid, with a ratio between (2.4-28.1) mg/g of fat, depending on the naturalness of the nutrition (Prandini et al., 2011).

Conjugated linoleic acid (CLA) is a name that refers to a group of conjugated dienoic positional and geometrical isomers of linoleic acid that are found in higher concentrations in ruminant milk and tissue fat than in other foods. It is noteworthy that the isomer Cis9Trans11 CLA is the most prominent among the isomers that have biological effectiveness in improving consumer health, CLA is formed as a result of the bio-hydrogenation of linoleic acid in the rumen by the action of microorganism's endemic in the digestive, these organisms have the ability to produce Linoleic Acid Isomerase enzyme (LA Isomerase), which convert linoleic acid into a number of isomers (Lin et al., 2003; Ares-Yebra et al., 2019; Song et al., 2021; Özer and Kılıç, 2021). Studies have tended to search for the bacterial strains that produce this enzyme. The ability of some strains of lactic acid bacteria to convert LA to CLA has been observed. Research indicated that the lactic acid bacteria, especially the genus *Lactobacillus* sp, have the ability to produce CLA, especially the Cis9Trans11 isomer, indicating that the enzyme system in this bacterium participates in the production of these isomer (Macouzet et al., 2009; Kishino et al., 2011; Ribeiro et al., 2018; Aziz et al., 2020). Accordingly, the study aimed to extract LA Isomerase enzyme from the bacteria *Lactobacillus paracasei* that produces CLA acid from different sources of milk, partially purifying the enzyme and studying the optimal conditions for the production of CLA.

2. Materials and Methods

Milk samples from cows and goats were collected from a breeder in Babel governorate - Iraq. As for breast milk, 15 samples were taken from healthy lactating mothers 10 days after birth. Samples were collected in sterile test tubes. The tubes were incubated at a temperature of 37 °C for 24 hours, to obtain coagulation and to dominance of the lactic acid bacteria, then transferred 1 ml from the coagulant tubes to tubes containing 9 ml sterile MRS Broth L-cysteine media. MRS Agar and Broth were designed to encourage the growth of the lactic acid bacteria which includes species of the following genera: *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc*. Lactic acid can be produced in large quantities by all of these species. They are Gram-positive, catalase and oxidase negative, and their nutritional requirements are stringent. Microaerobic conditions boost growth significantly. Lactic acid bacteria have a slower growth rate and a smaller colony size than other microorganisms. In non-selective media, they may overgrow, especially if incubation is required for 2-4 days. Although MRS medium is lactobacilli-specific, it can also support the growth of leuconostocs and pediococci.

Samples were incubated at 37 °C for 24 hours, the process was repeated 3 times to ensure purification of isolates.

2.1. Preparation of MRS media

De Man Rogosa and Sharpe (MRS) media were prepared with some modifications at concentration ($g.L^{-1}$) (Guo et al., 2018): Glucose (4.0), Beef extract (2.0), Peptone (1.0), Yeast extract (1.0), CH3COONa (1.0), C6H17N3O7 (0.4), Na2HPO4 (0.4), MgSO4 (0.24), MnSO4 (0.06) and C6H12O5 (2.0) and L-cysteine 0.05%, adjust pH at 6.2.

2.2. MRS agar tagatose

MRS agar was created primarily for the cultivation of lactobacilli from various sources with the goal of producing a defined medium that could be used instead of tomato juice agar. It is used to cultivate the entire lactic acid bacteria family. Although the medium is productive for nearly all lactic acid bacteria, the original version is not. Preparation of MRS Agar Tagatose media by substituting glucose sugar in MRS media with sugar D-Tagatose at 1% concentration (supplied by SIGMA). This experiment was conducted to obtaining strains of *Lactobacillus paracasei*, selected bacterial cultures were activated using tubes containing 9 ml of MRS Broth L-cystein culture media, 0.1 ml were transferred to MRS Agar D-Tagatose media, and spread well using L-Shap. The dishes were incubated under anaerobic conditions using a Gas pack (AnaeroGen, Oxoid Co. UK) for 24 h at 37 °C. The colonies' morphological and characteristics were studied.

2.3. Production of conjugated linoleic acid

The ability of the selected colonies to produce conjugated linoleic acid was studied by preparing tubes containing 9 ml of MRS Broth, 0.05%L-cystein, 1% Tween 80 and linoleic acid (LA) concentration of 0.2 mg/ml. The tubes were inoculated with selected bacterial isolates at a concentration of 2% and then incubated at 37 °C for 24 h under anaerobic conditions.

2.4. Estimation of conjugated linoleic acid

In estimating CLA with some modification, centrifugation was performed at 10,000 rpm/5 min for tubes incubated in the above paragraph (Liu et al., 2011). Transferred 3ml of the surface layer of tubes and add 6 ml of isopropanol and shake well for 1 min, then add 5 ml of hexane 98% (Hexan for HPLC, BDH, UK.), and shake well for 1 min.

The centrifugation was carried out at 5,000 rpm/5 min. at 4°C, then transferred 2 ml of these tubes to cuvette of Spectrophotometer (Apel, UK) and scan it at a wavelength ranging between 220-280 nm, noting that there is a peak at the wavelength of 233 nm, as it indicates the presence of CLA. The amount of CLA was calculated by making a stander curve for CLA with a concentration of (0-12) µg/ml dissolved in hexane and measuring the absorbance at the wavelength of 233 nm. As for the conversion rate, it was calculated through the Equation 1:

$$CLA = \frac{\text{amount of CLA from standrd Curve}}{\text{LA}} \times 100 \quad (1)$$

2.5. Isolates diagnosed with the VITEK 2 compact device

The VITEK 2 device (Biomerieux, USA) was used to diagnose isolates according to the instructions of Biomerieux Anonymous (BioMérieux, 2010). 1 ml of high yield isolates was transferred to sterile test tubes containing 3 ml of Normal Saline. For each tube, the turbidity of the bacterial suspension was determined by the turbidity meter (DensiChek ™) supplied with the VITEK 2 so that the turbidity of the suspensions was equal to (2.7-3.3 McF). The tubes containing the bacterial suspension were placed in a cassette within the incubator of the VITEK 2 device.

2.6. Extraction of enzyme

After diagnosing high-yielding bacteria of CLA, activated on MRS broth media, the crude enzyme was extracted from *Lactobacillus paracasei* isolate (Lin et al., 2003).

2.7. Assay of enzyme activity

The enzymatic activity was estimated according to the method described in Wu (2001). Depending on optical methods, prepare the reaction solution at a concentration of 24 µmol by mixing 0.1 mL of LA with 2.7 mL of phosphate buffer pH 7 with 0.2 mL of 1,3propanediol in a silica cell - incubate the mixture. At a temperature of 35 °C for 5 minutes, 0.1 ml of the enzymatic extract was added and left for 10 minutes. The absorbance was measured at a wavelength of 233 nm. The activity was estimated based on the standard curve for conjugated linoleic acid or from the straight-line equation. The enzyme unit was defined as the required amount that liberates 1 nmol of linoleic acid, which equates to the change in absorbance by 0.008 per minute, and the qualitative effectiveness is expressed as the enzyme unit per mg of protein.

2.8. Protein concentration estimation

Protein concentration was estimated according to Bradford (1976).

2.9. Extraction of conjugated linoleic acid

The CLA extraction, the absorbance was estimated at a wavelength of 233 nm and the conjugated fatty acid concentration was calculated using the aforementioned standard curve (Krieg and Holt, 1984).

2.10. Partially purification of the enzyme using ammonium sulfate

The enzyme was partially purified by using ammonium sulfate, with some modifications (Lin et al., 2003). the crude enzyme extract solution was taken and it was partially purified using ammonium sulfate at a rate of 30-65%. The solution was mixed on the magnetic stirrer (Stewart Co., UK) for 1 hour at a temperature of 4 °C, after which the solution was centrifuged at 10,000 rpm for 30 minutes at a temperature of 4 °C. The sediment was taken and dissolved in 30 ml of the sodium buffer solution at a concentration of 20 mM and pH 7. The solution was dialyzed against distilled water for 24 hours at 4 °C, then, the sediment was collected and determined the specific activity and protein concentration for it.

2.11. Optimal conditions for CLA production using partially purified enzyme

The partially purified enzyme was used to determine the optimum conditions for conjugated linoleic acid production.

- pH: The amount of CLA was quantified by photometric method at different pH ranges 4, 5, 6, 7, 8 at 35 °C for 60 minutes in the incubator;
- Temperature: The amount of CLA was estimated at different temperature ranges (30, 35, 40, 45, 50 and 55) °C and for an incubation period of 60 minutes after fixing the optimal pH by step 1;
- Enzyme concentration: Different concentrations of the enzyme were used, ranging (0, 0.1, 0.3, 0.5, and 0.7) ml. The amount of CLA was estimated after fixing pH, incubation period, and the optimum temperature for the enzyme;
- Concentration of the LA: Different concentrations of pure LA were used 0, 100, 200, 300, 400 and 500 µg/ml. Then, the amount of CLA was estimated after fixture each of the above conditions (pH, Temperature, conc. of LA).

2.12. Diagnosis of reaction products

Samples were diagnosed using Shimadzu GC/MS - PQ2010 Ultra gas chromatography technique connected to mass spectrometry in according to the following separation conditions: - Column type 30 M × 0.25 mm id, film thickness 0.25µm DP-5MS and helium gas was used as a carrier gas, with a flow rate of 1 ml/second, and the temperature of the injector and the interconnector was 280 °C. The GC oven program was set to an initial temperature of 100 °C for 1 minute, after which the oven temperature was raised to 280 °C at 6 °C/min, and the spectra of the curves were matched with the NIST 08 Spectral Library.

2.13. Statistical analysis

The experiment was designed using a completely randomized design (CRD), and the averages were compared according to LSD at P≤ 0.01significance level, with used three replicates for each treatment.

3. Results and Discussion

3.1. Morphological and biochemical characteristics of isolates

Milk possesses many components that make it the focus of attention of many researchers. It was used in the current study as a source for isolating bacteria because it is rich in fatty acids, especially CLA, which gives an indication of the ability of these bacteria to resist the toxicity of the conjugated fatty acid.

Table 1 shows the selected isolates on MRS D-Tagatose Agar media, as it was observed the colonies of bacterial were spherical, convex, cream to white, shiny, non-sticky, and equidistant. Negative for catalase and not hydrolysed of gelatin, as well as noted that 13 isolates were gas-producing, which indicates that these isolates are Heterofermentative, while the remaining 17 isolates were non-gas producing, obligately homofermentative. The reason for obtaining a good number of isolates of lactic acid bacteria may be attributed to se of the MRS media containing Sodium Acetate, presence of L-cysteine HCL that causes a reduction in the oxidative and reduction potential of the media as well as the anaerobic conditions for growth of bacteria. And leaving of milk samples in the incubation at 37ºC for 24 hours, produces rise in the acidity of samples due to the production of lactic acid by the action of lactic acid bacteria, because of dominance of these bacteria at the

Table 1. The morphological and biochemical characteristics of isolates, their susceptibility to CLA production, conversion rate and enzymatic activity.

% CLA	CLA	Gas Test	Gelatin Test	Catalase Test	Mycelial	Gram Stain	Color	Colony Shape	Isolate No.
	µg/ml				Morphology				
60.22	120.45[a]	+	-	-	Rod	G+	Milky White	Circular	H_1
53.89	107.78[c]	+	-	-	Rod	G+	White	Irregular circle	H_2
48.77	97 [d].55	+	-	-	Rod	G+	Milky White	Irregular circle	H_3
-	-	-	-	-	Rod	G+	Milky White	Irregular circle	H_4
-	Tolerated	-	-	-	Rod	G+	Milky White	Irregular circle	H_5
-	-	-	-	-	Rod	G+	Milky White	Circular	H_6
35.59	71 [g].19	+	-	-	Rod	G+	Milky White	Circular	H_7
-	-	-	-	-	Rod	G+	Milky White	Circular	H_8
-	-	-	-	-	Rod	G+	Milky White	Circular	H_9
-	Tolerated	-	-	-	Rod	G+	Milky White	Irregular circle	H_{10}
55.26	110 [c].52	+	-	-	Rod	G+	Milky White	Irregular circle	C_1
-	Tolerated	-	-	-	Rod	G+	white	Irregular circle	C_2
-	Tolerated	-	-	-	Rod	G+	Milky White	Circular	C_3
-	-	-	-	-	Rod	G+	White	Circular	C_4
39.05	78 [f].11	+	-	-	Rod	G+	Milky White	Circular	C_5
-	-	+	-	-	Rod	G+	Milky White	Irregular circle	C_6
-	Tolerated	-	-	-	Rod	G+	Milky White	Irregular circle	C_7
-	-	+	-	-	Rod	G+	Milky White	Irregular circle	C_8
-	-	-	-	-	Rod	G+	white	Irregular circle	C_9
-	-	-	-	-	Rod	G+	Milky White	Circular	C_{10}
57.61	115 [b].23	+	-	-	Rod	G+	White	Circular	G_1
-	-	-	-	-	Rod	G+	Milky White	Circular	G_2
-	-	-	-	-	Rod	G+	White	Irregular circle	G_3
54.73	109 [c].47	+	-	-	Rod	G+	White	Irregular circle	G_4
-	-	-	-	-	Rod	G+	Milky White	Irregular circle	G_5
44.28	88 [e].56	+	-	-	Rod	G+	White	Circular	G_6
-	-	-	-	-	Rod	G+	White	Circular	G_7
-	-	-	-	-	Rod	G+	Milky White	Circular	G_8
29.84	59 [h].68	+	-	-	Rod	G+	Milky White	Circular	G_9
32.64	65 [h].29	+	-	-	Rod	G+	Milky White	Irregular circle	G_{10}

H = Human milk; C = Cow Milk; G = Goat Milk.

expense of other types of bacteria. These results are in agreement with what was mentioned in Bergy's Manual of Systematic Bacteriology (Krieg and Holt, 1984). A study also indicated that breast milk is a good source of lactic acid bacteria in the intestines of children, which acts as an anti-infection and improves immunity and metabolism (Fernández et al., 2013).

Table 1 also showed the ability of some bacteria isolated from human milk, cows and goats to produce CLA acid in MRS Broth L-Cystein HCL media with 0.2 mg/ml LA acid added during incubation at 37 °C for 24 hours, as 11 isolates managed to produce CLA acid at rates varying between (120.45-59.68) µg/ml, with a conversion rate of (60.22-29.84) %. As three isolates sourced from mother's milk were obtained: H_1, H_2 and H_3 that have the ability to produce CLA at a concentration of (120.45, 107.78 and 97.55) µg/ml, with a conversion rate (60.22, 53.89 and 48.77%), Whereas, C_1 and C_5 isolates from cows' milk showed the ability to produce CLA with a concentration of 110.57 and 78.11 µg/ml with a conversion rate of (55.26 and 78.11)%, While five isolates (G_1, G_4, G_6, G_9 and G_{10}) from goat's milk were able to produce CLA at concentrations ranging between (115.23-59.68) µg/ml with a conversion rate of (57.61-29.84)%. The ability of some isolates to produce CLA at varying concentrations may be attributed to the ability of these bacteria to secrete the enzyme LA Isomerase, which works to convert LA into CLA, thus reducing the toxicity of LA acid to bacterial isolates in the media. Also, some isolates were able to grow without producing acid, it may be attributed to their ability to tolerate the concentration of LA acid in the media due to their functional characteristics and isolation sources that gave these bacteria the ability to grow in a cultural media containing LA.

As for the rest of the isolates that did not show growth in the presence of LA in the culture media, it may be attributed to the adsorption of acid on the cell wall, causing changes in cell permeability, in whole or in part. These results were in agreement with Ares-Yebra et al. (2019), observed the ability of *L. plantarum*, *L. acidophilus* and *L. casei* bacteria to produce CLA in the culture media MRS broth and the skimmed milk that added 1 mg/ml from LA. Ogawa et al. (2005) stated that the production of CLA varies from one bacterium to another, as he indicated that the productivity of *Bifidobacterium* bacteria ranges between 3.5-350 µg/ml, while *Lactobacillus* bacteria produce concentrations of CLA of (20-4900) µg/ml. Some studies have indicated that CLA cannot be produced without the presence of LA as a reactive media (Ares-Yebra et al., 2019).

3.2. Diagnosis of bacterial isolates

The diagnosis of high-yielding isolates of CLA represented by H_1, G_1 and C_1 was made using the VITEK device, as the examination was with the VITEK 2 compact device of 64 different sugars and enzymes, through the stages in which the sample passes. Where it was noticed that the isolates belonged to the genus *Lactobacillus*, as H_1 and C_1 showed that they belong to *L. paracasei* bacteria with a probability of 89%, While G_1 isolate is *L. plantarum* with 95% probability, as it appears in the report of the device (Figure 1).

3.3. Enzymatic activity

The high-yield H1 isolate of CLA was selected for enzyme extraction. The concentration of CLA produced in the reaction medium was estimated using colorimetric methods (Table 2). As the concentration of CLA in the media of the reaction to which the crude enzyme extract was added reached 22.3 µg.ml^{-1} with a conversion rate of 22.3%, while this percentage increased to 35.7 µg.ml^{-1}with a conversion rate of 35.7% when using the enzyme LA Isomerase partially purified by using sulfates, the reason for the increase of production may be attributed to the role of ammonium sulfate to removal of some compounds that may prevent the binding of the substance with the enzyme active site. Lin et al. (2003) found that the highest yield of CLA was obtained when LA Isomerase was added at 50 mg protein.

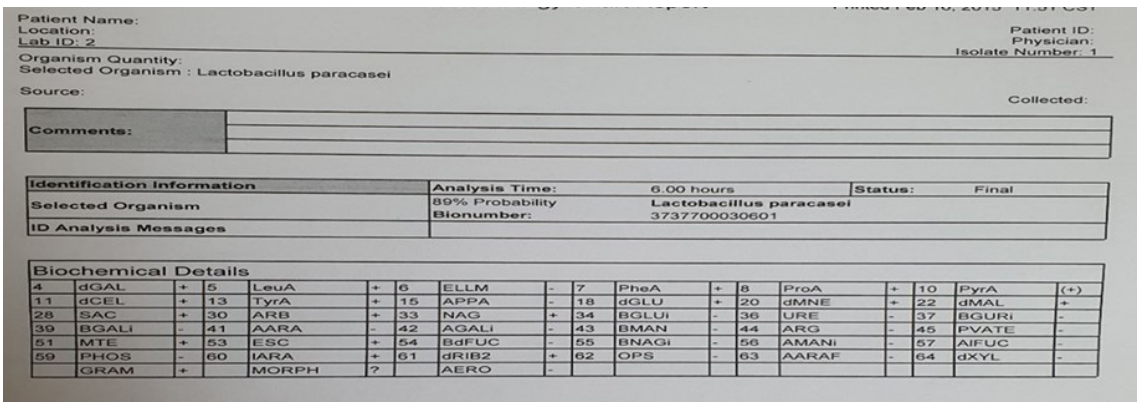
3.4. Optimum conditions for CLA production

3.4.1. pH

Table 3 shows the concentration of CLA at different pH ranges in the reaction media added to it with 100 µg.ml^{-1}of standard LA at a temperature of 35 °C, as a gradual increase in the concentration of the conjugated acid was observed

Table 2. CLA concentration and conversion ratio for the crude and partially purified enzyme of L. paracasei isolate.

% Conversation	CLA µg.ml^{-1}	Enzyme
22.3	22.3[a]	Crude Enzyme
35.7	35.7[b]	Part. Purified Enzyme


Patient Name:
Location:
Lab ID: 2
Patient ID:
Physician:
Isolate Number: 1
Organism Quantity:
Selected Organism : Lactobacillus paracasei
Source:
Collected:
Comments:

Identification Information	Analysis Time:	6.00 hours	Status:	Final
Selected Organism	89% Probability	Lactobacillus paracasei		
ID Analysis Messages	Bionumber:	3737700030601		

Biochemical Details

4	dGAL	+	5	LeuA	+	6	ELLM	-	7	PheA	+	8	ProA	+	10	PyrA	(+)
11	dCEL	+	13	TyrA	+	15	APPA	-	18	dGLU	+	20	dMNE	+	22	dMAL	+
28	SAC	+	30	ARB	+	33	NAG	+	34	BGLUi	-	36	URE	-	37	BGURi	-
39	BGALi	-	41	AARA	-	42	AGALi	-	43	BMAN	-	44	ARG	-	45	PVATE	-
51	MTE	+	53	ESC	+	54	BdFUC	-	55	BNAGi	-	56	AMANi	-	57	AIFUC	-
59	PHOS	-	60	IARA	+	61	dRIB2	+	62	OPS	-	63	AARAF	-	64	dXYL	-
	GRAM	+		MORPH	?		AERO	-									

Figure 1. Report of the VITEK 2 device.

with the rise in the pH of the reaction media to neutral, as it increased from 13.21 µg.ml^{-1} at pH 4.5, reaching its maximum output at pH 6.5, Production decreased sharply in the base media, as it reached its maximum decrease at pH 8.5 reaching 2.77 µg.ml^{-1}. The reason for the decrease could be attributed to partial denaturation of the enzyme active site As for the variation in the production of the conjugated fatty acid in the results of the current study, it may be due to the fact that the enzymes are sensitive to changes in pH through their work in a limited range, as the change in pH effects on the ionic state of the amino acids present in the enzyme and the molecules of the substrate, Which may affect negatively or positively on the efficiency of binding the subject substance to the active site of the enzyme, these effects may lead to a change in the active site of the enzyme.

Wu (2001) found that the highest CLA production was obtained from the interaction of the isomerase enzyme extracted from *Lactobacillus acidophilus* with LA at pH between (6-7), while observed a sharp decrease in production at pH 8. Lin et al. (2003) found that the highest production of CLA was obtained at pH 5 and 7, as it reached (1700 and 1099) µg.ml^{-1} from the reaction of the enzyme LA Isomerase enzyme extracted from *L. acidophilus* bacteria and *Propionibacterium freudenreichii* ssp. *shermanii*, respectively

3.4.2. Temperature

Table 4 showed the effect of the temperature of the reaction media on the production of CLA, as the results showed that the highest concentration of the conjugated fatty acid was obtained at a temperature of 45°C. It reached 42.72 µg.ml^{-1}, with a conversion rate of 42.72%, and with the increase in the temperature a sharp decrease in the concentration of CLA was observed. It reached (23.2 and 9.14) µg.ml^{-1} at the temperature of (50 and 55) °C, respectively. The reason for the gradual increase in production between 30-45°C may be attributed to the increase in collisions between the enzyme molecules and the substrate, which leads to an increase in the speed of the enzymatic reaction (Segel, 1975). The sharp decrease in CLA formation may be attributed to the effect of the active site in the enzyme by high temperatures. Partial purification of the enzyme gave preference to the production of CLA despite the high temperatures.

Observed an increase in enzymatic activity with an increase in the temperature of the reaction medium, but at a certain temperature range. However, continuing to increase the temperature of the reaction medium may lead to a decrease in CLA production or a cessation of its production due to loss of secondary and tertiary structure of the enzyme or partial or complete denaturation of the active site. Also, Wu (2001) indicated that the best temperature for the action of Isomerase extracted from *L. acidophilus* was 37 °C when used to produce CLA in the reaction medium. While Lin et al. (2002) used a temperature of 50°C for the reaction media consisting of linoleic acid and the Isomerase enzyme extracted obtained from *L. acidophilus* and *Propionibacterium freuderreichii ssp. Shermanii*. Lin et al. (2003) indicated that a temperature of 50°C was used in the production of CLA by mixing the enzymatic extract of *L. acidophilus* CCRC14079 with linoleic acid.

3.4.3. Concentration of substrate

The effect of the substrate concentrations (100 - 500) µg.ml^{-1} of LA on the production of CLA was studied in the presence of the enzyme extract of *L. paracasei* bacteria isolated from breast milk, and as shown in Table 5, A slight change was observed in the production of the conjugated fatty acid with an increase in the concentration of the substrate, as it reached its maximum produce at the concentration of 500 µg.ml^{-1} of LA in the media of the reaction, the CLA production was reached 46.79 µg.ml^{-1} with a conversion rate of 9.358%, While no significant change was observed in the other concentrations, the reason may be attributed to the occurrence of saturation in the enzyme active sites. It should also be noted that the highest conversion rate was obtained when adding 100 µg.ml^{-1} of LA to the reaction media, at conversion rate 31.74%.

For a study using two concentrations of 75 and 50 mg of LA as a control substance in CLA production, Lin et al. (2003) that concentrations of 75 mg of LA gave the highest yield of conjugated fatty acid compared to the concentration of 50 mg. Kim et al. (2000) noted that Isomerase enzyme extracted from the bacterium *Butyrivibrio fibrisolvent* A38 has not been recycled like the natural enzymes to stimulate it to interact with the substrate.

As the production of CLA depends to a large extent on the density of the bacteria cells that work to produce the enzyme. Wu (2001) also indicated that the speed of the reaction increases with the increase of the substrate until the maximum (maximum velocity) of the reaction is approached, when the presence or addition of concentrations of the substrate does not lead to enhance

Table 3. Effect of different pH ranges in CLA production.

PH					Isolate
8.5	**7.5**	**6.5**	**5.5**	**4.5**	
2.77^{e}	10.11^{d}	31.74^{a}	22.42^{b}	13.21^{c}	CLA µg.ml^{-1}
2.77	10.11	31.74	22.42	13.21	% Conversation

Table 4. The effect of temperature on CLA production.

Temp. °C						Isolate
55	**50**	**45**	**40**	**35**	**30**	
9.14^{f}	23.2^{d}	42.72^{a}	37.44^{b}	31.74^{c}	25.81^{d}	CLA µg.ml^{-1}
9.14	23.2	42.72	37.44	31.74	25.81	% Conversation

the speed of the reaction and thus does not cause an increase in the conjugate fatty acid production.

3.4.4. Enzyme concentration

Table 6 shows the use of different concentrations ranging between (0.1 - 0.7) ml of the enzymatic extract of *L. paracasei* bacteria and partially purified in the production of CLA. As a gradual increase in the amount of the conjugated fatty acid with an increase in the concentration enzymatic extract was observed, as the maximum CLA production at a concentration of 0.7 ml reached 55.42 µg/ml, and the reason for this increase may be due to the abundance of the active site for its association with the substrate and failure the reaction to reach the maximum speed, which enabled the continuation of the enzymatic reaction in the production of the conjugated fatty acid. Lin et al. (2002) observed that the highest CLA production was obtained from the application of 20 mg of the enzymatic extract of *L. acidophilus* and *Propionibacterium freudenreichii ssp. shermanii* CCRC11076. Lin et al. (2003) found that the use of concentrations of (0, 25, 50 and 75) mg of the enzymatic extract of *L. acidophilus* bacteria gradually increased the production of linoleic acid conjugated with an increase in the concentration of the added enzyme, as the results showed for the conjugated acid 75 mg protein.

3.5. Diagnosis of enzymatic reaction with GC-MS technique

Figure 2 shows the diagnosis of the reaction products of LA Isomerase with linoleic acid by GC-MS technique. It

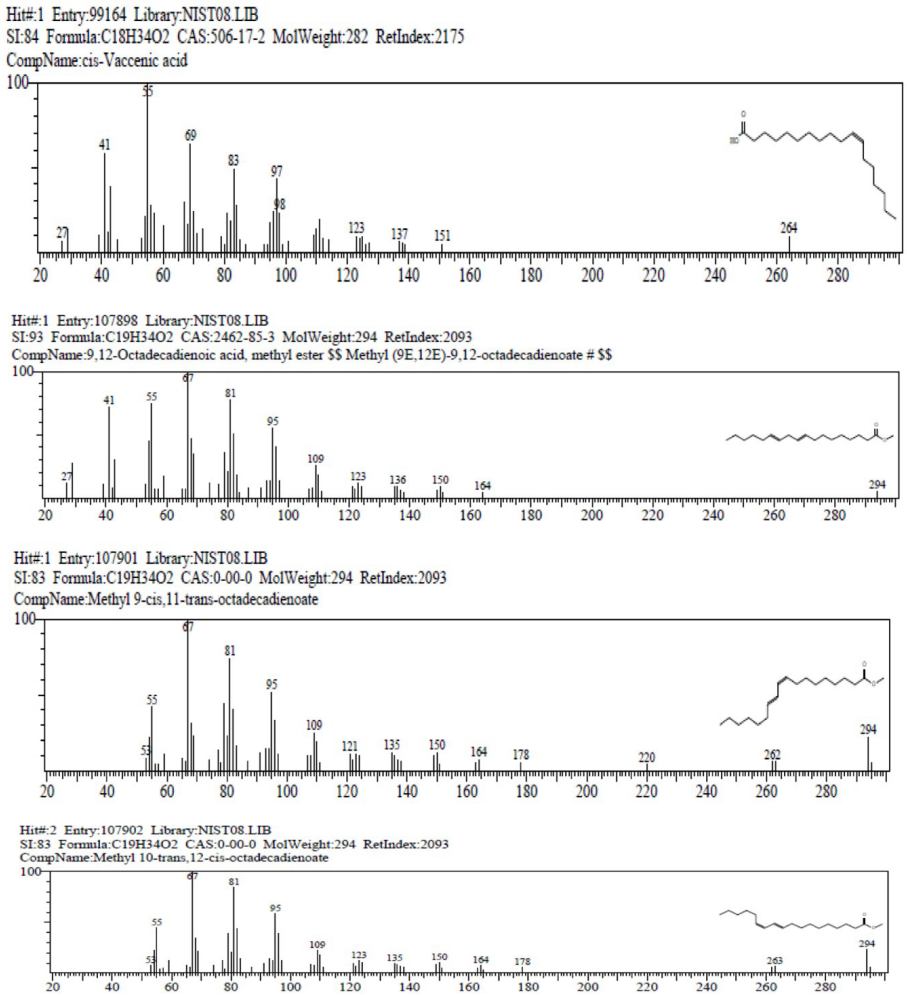


Figure 2. Diagnosis of the enzymatic products with GC/MS technology.

Table 5. The effect of the substrate on the production of CLA.

Substrate µg/ml						
500	**400**	**300**	**200**	**100**	**0**	**Substrate µg/ml**
46.79[a]	45.88[a]	44.7[a]	44.75[a]	31.74[b]	0	CLA µg/ml
9.358	11.47	14.9	22.375	31.74	0	% Conversation

Table 6. The effect of concentration the partially purified enzymatic extract in the production of CLA.

Conc. of enzyme (ml)					
0.7	**0.5**	**0.3**	**0.1**	**0**	**Conc. of enzyme(ml)**
55.42[a]	43.66[b]	35.2[c]	31.74[d]	0	CLA µg/ml
55.42	43.66	35.2	31.74	0	% Conversation

was observed that there were a number of isomers of the conjugated fatty acid were (C_9T11, T_9T_{12} and $T_{10}C_{12}$), as well as observed the presence of Vaccenic Acid, which is the basic acid for the formation of CLA isomers. These results agreed with the findings of Sosa-Castañeda et al. (2015) noting the ability of a number of strains of *Lactobacillus* bacteria to produce isomers from the conjugated fatty acid CLA, as these products included ($T_{10}C12$-C_9T_{11}) - CLA. Lin et al. (2003) found that adding LA Isomerase enzyme produced from *Lactobacillus acidophilus* CCRC 14079 to the reaction medium in the presence of LA produced a number of isomers (T_8T_{10}, T_9T_{11}, $T_{10}T_{12}$, $T_{11}T_{13}$, T_8C_{10}, C_9T_{11}, $T_{10}C_{12}$, $C_{11}T_{13}$) -CLA in various concentrations.

4. Conclusion

Human milk has a unique composition and is only recommended for children under the age of 6 months who require complete nutrition. Apart from meeting nutritional needs, it also provides an immediate source of protective factors and aids in the development of the immune system, both of which are critical for a child's protection. Human milk oligosaccharides (HMOs), which are 'prebiotics' that encourage the colonization of beneficial 'probiotic' bacteria in the gut, are among these factors. The current study showed the ability of a number of Lactobacilli bacteria to produce CLA at different concentrations, in which the Lactobacilli bacteria obtained from breast milk, As the Isomerase enzyme was extracted from these bacteria and was partially purified, then used it in the production of conjugated fatty acid. When studying the optimal conditions for the enzymatic reaction with substrate, the GC-MS technique showed the presence of a number of reaction products that are isomers of conjugated linoleic acid (C_9T_{11}, T_9T_{12}, $T_{10}C_{12}$), with different concentrations. The results of the GC-MS diagnostics also showed the presence of a number of isomers for conjugated linoleic acid.

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