Extraction, Purification and Characterization of Cholesterol Oxidase Enzyme Biosynthesized by Probiotic *Lactiplantibacillus plantarum* MF1

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Abstract

This work concerned with purification and characterization of the cholesterol oxidase enzyme synthesized by probiotic bacteria insulated from raw cow's milk. The bacterial isolate was genotyped as *Lactiplantibacillus plantarum* by 16S rRNA and deposited with GenBank under accession number MW242720. The *L. plantarum* MF1 cholesterol oxidase enzyme was purified using acetone and sephadex G 100 column chromatography, yielding enzyme recovery and purification folds of 19.3% and 4.2, respectively. The purified cholesterol oxidase appeared as a single protein band at 51 kDa using SDS-PAGE technique. For maximum activity of the purified CHO enzyme the optimal conditions were a 30 minutes' incubation period at 30°C, a substrate concentration of 150 µL in the reaction mixture, and an enzyme concentration of 100 µL in the reaction mixture. The purified cholesterol oxidase was activated by metal ions (CuSO4), whereas it was inhibited by $CaCl_2$, $MgSO_4$, $ZnSO_4$, and $MnCl_2$. After storage at -20°C for 4 days, the purified enzyme retained 57% of its initial enzyme activity. The purified enzyme demonstrated an active effect on serum human cholesterol samples, resulting in a 29% reduction.

Key words: *Lactiplantibacillus plantarum* MF1, Cholesterol oxidase enzyme, GenBank.

Introduction

Coronary artery disease is the main cause of mortality in many countries worldwide **(*Rhee et al., 2019*)**, and the global prevalence of cardiovascular diseases is increasing **(*Townsend et al., 2016*)**. Hypercholesterolemia increases the risk of heart attack by three times compared to people with normal blood lipid profiles **(*Kumari and Kanwar, 2012*)**. Probiotics are

gaining significance due to their innumerable benefits, such as the treatment of hypercholesterolemia, lactose intolerance, cardiac diseases, and the management of cardiac problems, including arteriosclerosis and atherosclerosis. To achieve the desired health benefit, health professionals advise their patients to use probiotics for prophylactic and therapeutic purposes **(*Maity and Misra, 2009*)**. Some bacteria and yeast genera have been identified as probiotics. The most commonly used are *Bifidobacterium*, *Lactobacillus*, and *Saccharomyces*. Despite its widely reported benefits, selecting the most effective probiotic strains has proven to be a difficult task that necessitates considering both efficacy and safety concerns **(*Rodríguez and Cardozo, 2012*)**. Probiotic microorganisms can grow by depending on the cholesterol as the only carbon source. Generally, these microorganisms synthesize an enzyme known as cholesterol oxidase (CHO) **(*Sanjeev et al., 2018*).** Cholesterol oxidase is a FAD-containing enzyme which relates to the family of oxido - reductases and catalyzes the initial step of cholesterol decomposition. In a step requiring FAD, this multifunctional enzyme converts cholesterol into cholest-5-en-3-one, which is isomerized to cholest-4-en-3-one with releasing of H_2O_2. It has been reported that many microorganisms have the potential to synthesize CHO. The interpretation of cholesterol-degrading microorganism isolation and characterization, as well as their ability of cholesterol decomposition, has a wide range of applications**.** The present study aims to extract, purify, and characterise the cholesterol oxidase enzymes from probiotic *Lactiplantibacillus plantarum* MF1.

Materials and Methods

Fourteen isolates of bacteria from milk products were gathered from supermarkets and farmers in Zagazig, City, Egypt, during the period from January to May 2019 and incubated in Minimal Salt Cholesterol (MSC) broth containing 0.2 % cholesterol as the sole carbon and energy source **(*Nishiya et al., 1997*)**, containing (g/L): NH_4NO_3, 17; $MgSO_4.H_2O$, 0.25; $FeSO_4.H_2O$, 0.001; NaCl, 0.05; K2HPO4, 0.25; cholesterol, 1.5 and Tween 80 (0.1 mL/L). To select the most cholesterol-lowering isolate, the pH was adjusted to 7.0. The bacterial culture broth was centrifuged at 5000 rpm for 15 minutes. After being sterilized with a 0.45 mm filter (Germany), the cell free filtrate was kept at -20°C until it was used. The potential of the bacterial isolates to degrade cholesterol was assessed using an enzymatic colorimetric (CHO)-(POD) method **(*Kulkarni et al., 2013*)**.

Identification of the most active cholesterol reducer isolate

According to Bergey's Manual of Systematic Bacteriology, the most active cholesterol reducer isolate was identified (***Krieg and Holt, 1984***) and at the species level at the Animal Health Research Institute's Biotechnology Unit, Dokki, Giza, Egypt. PCR was used to amplify the 16S rRNA by using the universal primers of bacteria 27 F (5′AGAGTTTGATCMTGGCTCAG-3′) and 1492 R (5′TACGGYTACCTTGTTACGACTT-3′). For identification the following steps were performed as follows: The PCR reaction began with a denaturation step for 5 minute at 94°C, following that 35 cycles of 94°C for 30 seconds, 58°C for 1 minute, and 72° C for 1-2 minutes. Finally, the extension step at 72°C for 12 minute. The PCR products were assured on 1% (w / v) agarose gels stained with ethidium bromide. The purified PCR products were placed in the sequencer (Biosystems 3130 genetic analyzer, USA). The CLUSTAL Omega program was used to align the sequence data. Using the GenBank nucleotide database and the BLAST Programme offered by the National Center for Biotechnology Information (NCBI), the 16S rRNA gene sequences were aligned to the nearest homologue. MEGA6 software was used to construct the phylogenetic tree including the partial 16S rRNA gene sequences from the closest type of strain **(*Tamura et al., 2013*)**.

Enzyme Production

After the fermentation process of the *L. plantarum* MF1 was ended, the bacterial pellet was separated from the cultural broth medium by filtration through filter paper (Whatman NO.1), then the filtrate was centrifuged for 10 minutes in a cooling centrifuge at 5000 rpm, and the clear cell free filtrate was applied as a crude enzyme.

Enzyme Activity Assay Method

Richmond method was used to measure the activity of cholesterol oxidase. The reaction was conducted at 30^{o}C for 30 minute using the following reaction mixture: 3 ml of 0.1 M sodium phosphate buffer (pH 7) with 0.05% Triton X-100, 0.05 ml clear supernatant, 0.05 ml of 6 mM cholesterol in 2-propanol. At 240 nm the increase in absorbance was measured by UV spectrophotometer **(*Richmond, 1976*).**

The equation used to determine the cholesterol oxidase activity was:

(ΔA × reaction volume × 0.082)/volume of enzyme used) = ΔA × 5.1 Uml−1.

One unit of enzyme equivalent the quantity of cholesterol oxidase that required to convert 1 µ mole of cholesterol to 4-cholesten-3-one at 30^{o}C per 1 minute. Bradford method was used to estimate protein content **(*Bradford, 1976*).**

Purification of enzyme

First, the enzyme was partially purified by precipitation with organic solvent (acetone). Different acetone to broth ratios, such as 1:1, 1:2, 1:3, and 1:4, were used for

precipitation, then resuspended the precipitated enzyme in phosphate buffer, enzyme activity and protein estimation were performed **(*Ghasemian et al, 2009*)**, and the most active enzyme (partially purified) was applied to the gel chromatography column (Sephadex G-100). At 1 ml/minutes, 58 fractions were collected. Each fraction's enzyme activity and protein content were determined separately. The determination of the purified *L. plantarum* MF1 cholesterol oxidase was performed by using of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS – PAGE) in Enzymology and Fungal Biotechnology lab (EFBL) of Botany and Microbiology Department, Faculty of Science, Zagazig University 44519, Egypt.

Characterization of the purified enzyme

The effect of different reaction mixture temperature (20-60˚C), reaction times (30 to 150 minutes), different concentrations of the enzyme (25-200 µL/reaction mixture), different cholesterol concentrations (25-200 µL/reaction mixture), different metal ions ($CaCl_2$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, and $MnCl_2$) and storage periods (0-8 days) on the purified enzyme activity were estimated.

Applications of the purified cholesterol oxidase

The purified *L. plantarum* MF1 cholesterol oxidase was tested to determine its impact on serum cholesterol **(*Doukyu, 2009*)**. After measuring the initial serum cholesterol levels, serum was separately treated with 5 µl purified enzyme and incubated at 37°C for 10 minutes. Following the kit's instructions, the amount of residual cholesterol was determined at the end of the incubation time (CHOD-PAP method, Spinreact Diagnostics, Spain).

Statistical analysis

The data obtained for three replicates of the parameters being studied were used to calculate the standard deviation, and one-way ANOVA was then used.

Results

Isolation and screening of cholesterol reducer isolate

The isolated and purified 14 probiotic bacteria were screened to compare their effect on the reduction of cholesterol. The result showed that bacterial isolate No.8 isolated from raw cow milk was the most active to reduce cholesterol by (33.07%)**.**

Molecular identification of the most cholesterol reducer isolate

The most potent cholesterol reducer isolate No.8 was biochemically identified as *Lactobacillus* sp (Table 1). The species were determined by 16S rRNA sequencing at the Animal Health Institute in Giza, Egypt. The determined 16S rRNA gene sequences were compared to the sequence available in the GenBank database

(http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTN program. When the 16S rRNA gene sequences of the isolate (No.8) were compared in GenBank, it showed 89.21% homology with *Lactobacillus plantarum* 1873 strain (accession number MT597698). Accordingly, isolate (No.8) was determined as a new strain of *L. plantarum* MF1 (Gen- Bank Accession No. MW242720). (Figure 1) demonstrates agarose gel electrophoresis of the partial sequences 16S rRNA gene of DNA of *L. plantarum* MF1, while the phylogenetic tree is depicted in (Figure 2).

Enzyme production and purification

The production of CHO was assessed quantitatively and CHO was (partially) purified by solvent extraction in an acetone to broth ratio (3: 1). The activity of the enzyme (partially purified) was recorded to be 3.06 U/mL in comparison with crude, which was 0.58 U/mL (Figure 3). The purification of CHO (Table 2) revealed a significant purification fold (4.2). (Figure 4) demonstrates the profile of *L. plantarum* MF1 cholesterol oxidase purification using sephadex G-100 chromatographic column. The electrophoresis result (Figure 5) indicated that the CHO molecular weight is about 51 kDa, and is represented by one band.

Characterization of CHO enzyme extracted *L. plantarum MF1*

The enzyme purified from *L. plantarum* MF1 reached its maximum activity at 30^{o}C after 30 minutes of reaction (Figure 6, 7). The results revealed that the optimum enzyme concentration was 100 µL in the reaction mixture. Nevertheless, the highest enzyme activity was obtained with a substrate concentration of 150 µL in the reaction mixture (Figure 8, 9). Metal ions ($CuSO_4$) activated the purified cholesterol oxidase, whereas $CaCl_2$, $MgSO_4$, $ZnSO_4$, and $MnCl_2$ inhibited it (Figure 10). The purified CHO enzyme was able to retain about 57 % of its initial activity after storage at −20 °C for 4 days (Figure 11).

Purified CHO applications

The addition of CHO enzyme extracted from *L. plantarum* MF1 to the serum of fifty samples led to reduction in cholesterol in all tested serum samples, as displayed in (Table 3) and (Figure 12).

Table (1) *Morphological and Biochemical Tests (manual) for (No.8):*

Character	Result
Cell shape	**Rod**
Colour colony	**White**
Aerobic/Anaerobic growth	**Facultative anaerobic**
Gram's reaction	**+ ve**
Catalase test	**- ve**
Indol	**- ve**
Citrate	**- ve**
Urease	**- ve**

Table (2): *Summary of purification steps of cholesterol oxidase enzyme from L. plantarum MF1:*

Purification steps	Total Enzyme activity (U/mL)	Total Soluble protein (mg/mL)	Specific activity (U/mg)	Recovery (%)	Purification fold
Culture filterate	**580**	**479**	**1.21**	**100**	**1**
Acetone(1:3)	**122.4**	**27.2**	**4.5**	**21.1**	**3.7**
Gel-filteration sephadex G-100	**112**	**22**	**5.09**	**19.3**	**4.2**

Table (3): *The Effect of CHO Enzyme Extracted from L.plantarum MF1 on serum samples:*

Before adding enzyme					**After adding enzyme**					**Mean of reduction ratio%**
Case	**No of Samples**	**Min**	**Max**	**Mean**	**Case**	**No of Samples**	**Min**	**Max**	**Mean**	
H	**15**	**246**	**332**	**279.8**	**H**	**1**	**252**	**252**	**252**	**23.6**
B	**13**	**208**	**239**	**223.3**	**B**	**12**	**200**	**229**	**214.9**	**23.8**
N	**22**	**150**	**198**	**167.9**	**N**	**37**	**108**	**187**	**146**	**23.58**

The risk evaluation: Normal (N): less than 200 mg/dl; Borderline(B): 200-239 mg/dl; High (H): more than or equal 240 mg/dl

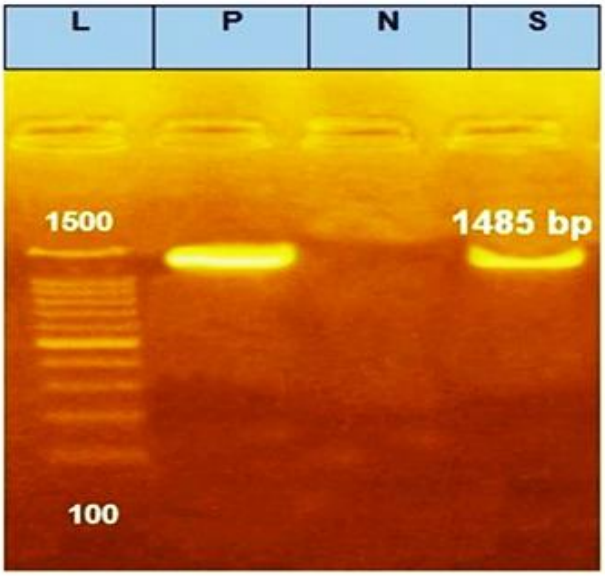

Figure (1): Agarose gel electrophoresis of 16S rDNA gene .

Lanes: l; DNA Ladder, P; positive, N; negative, S; *L. plantarum* MF1

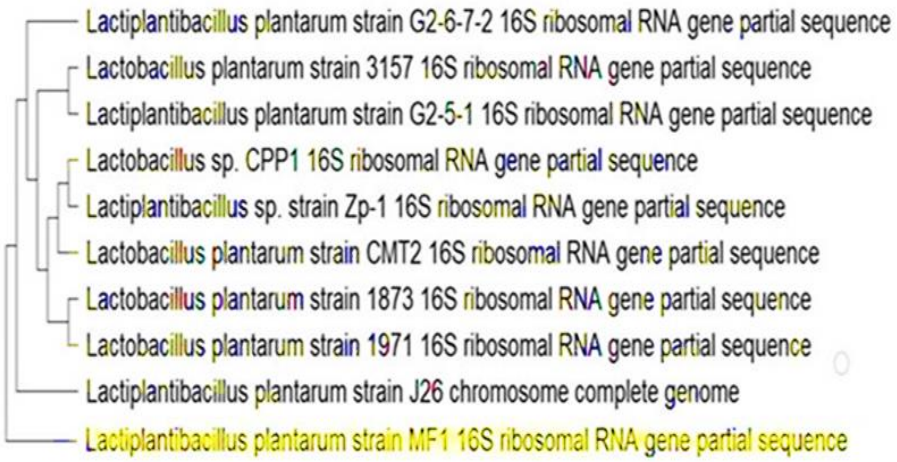

Figure (2): Phylogenetic Tree of *L. plantarum* MF1

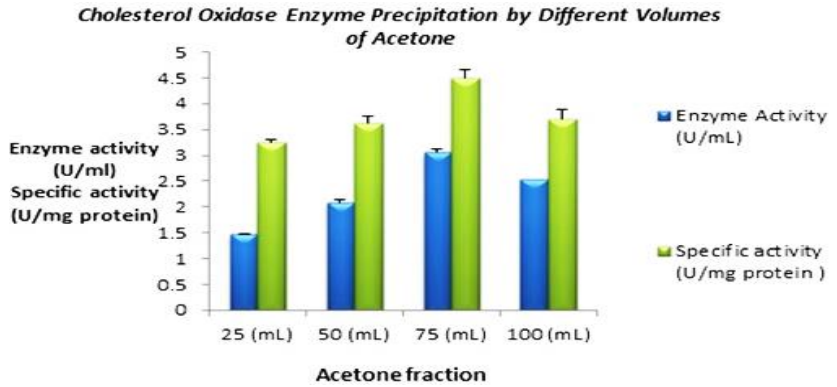

Figure (3): Precipitation of cholesterol oxidase enzyme by different volumes of acetone

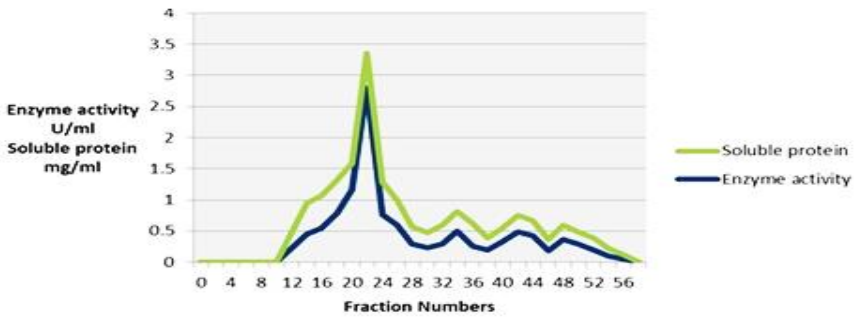

Figure (4): Purification of cholesterol oxidase from *L. plantarum MF1* by sephdax G – 100 chromatographic columns.

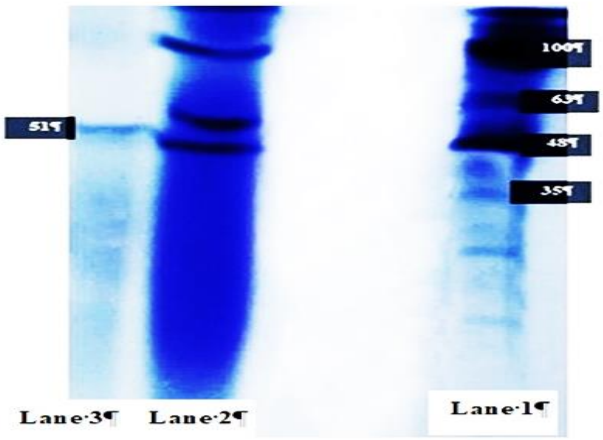

Figure (5): SDS-PAGE of purified CHO enzyme of *L. plantarum* MF1 growing in basal cholesterol liquid medium.
Lane 1: protein marker. Lane 2: crude enzyme
Lane 3: band of CHO enzyme of *L. plantarum* MF1 from Sephadex G-100.

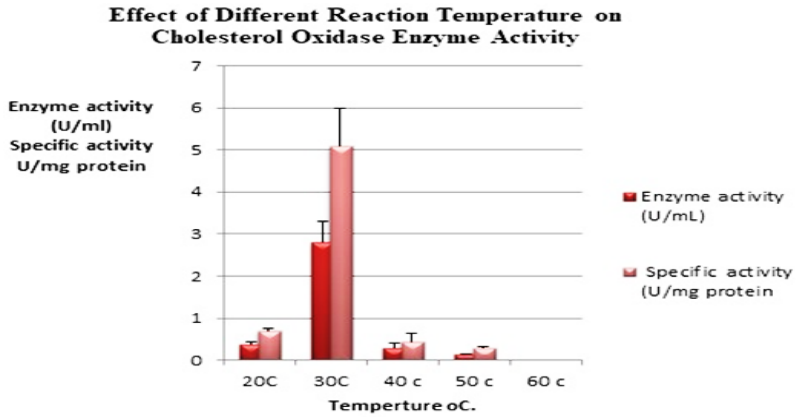

Figure (6): The effect of different reaction temperature on CHO specific activity of *L. plantarum* MF1.

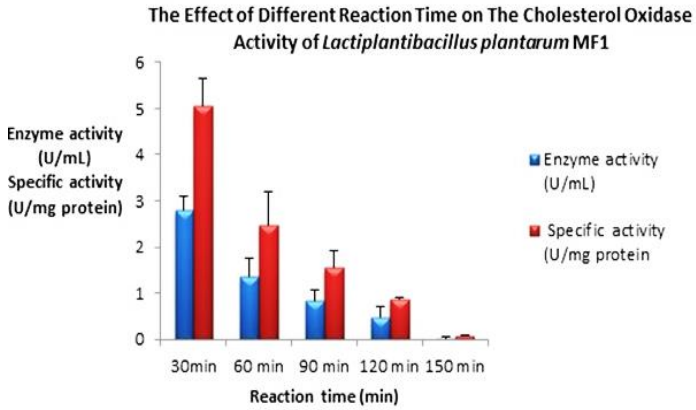

Figure (7): The effect of different reaction time on CHO enzyme from *L.plantarum* MF1

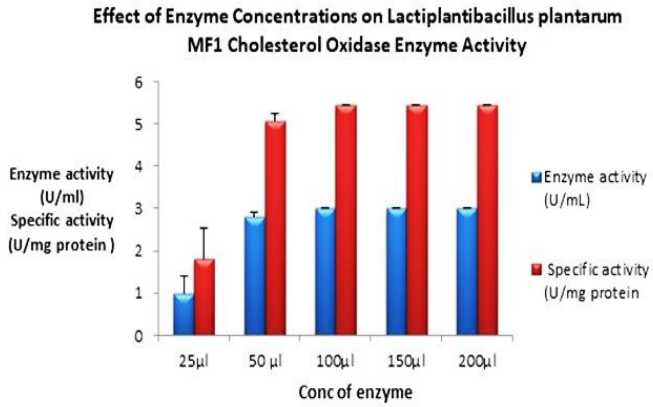

Figure (8): Effect of The effect of enzyme concentration on CHO enzyme from *L. plantarum* MF1

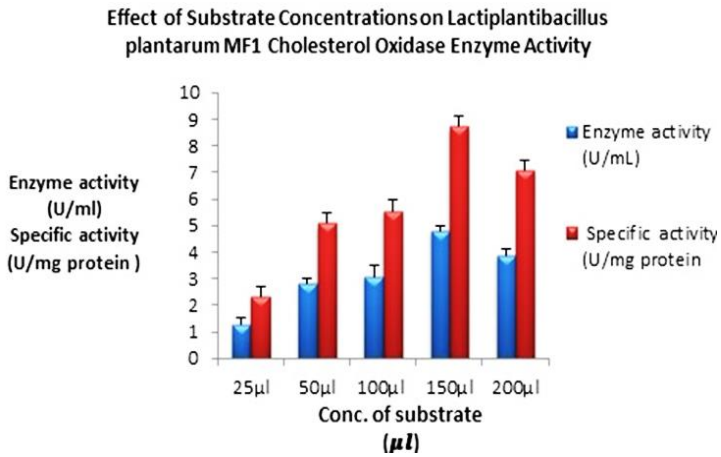

Figure (9): Effect of The effect of substrate concentration on CHO enzyme from *L.plantarum* MF1

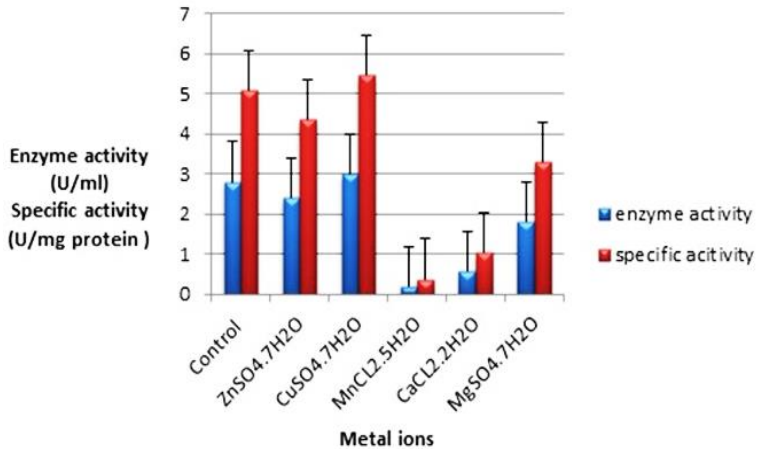

Figure (10): Effect of some metallic ions on activity of CHO enzyme from *L.plantarum* MF1.

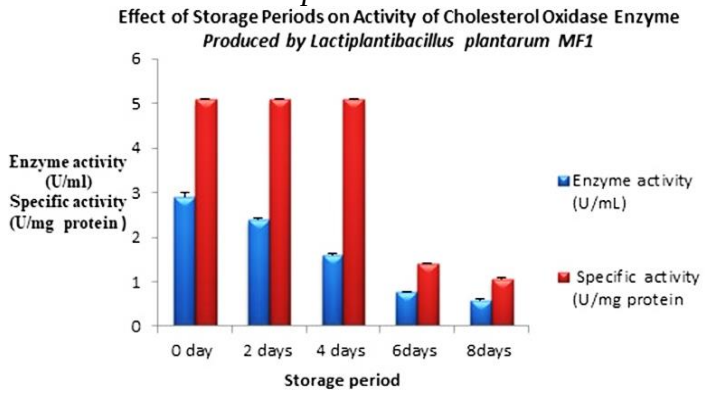

Figure (11): Effect of different storage periods on CHO enzyme from *L. plantarum* MF1

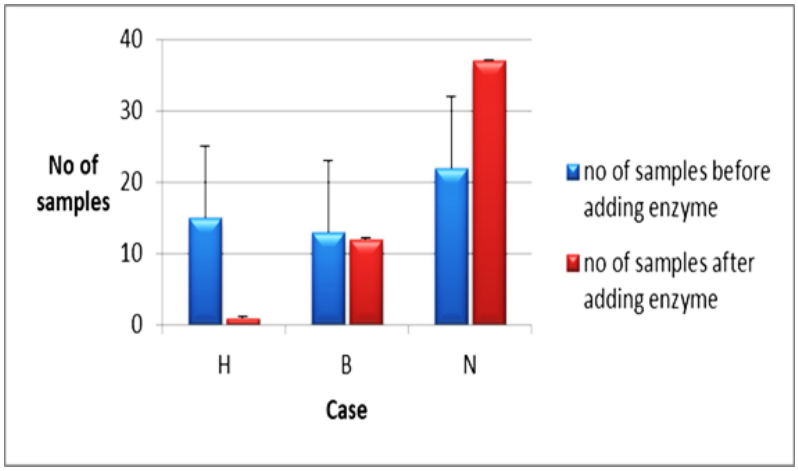

Figure (12): Summary of the impact of purified CHO enzyme on serum human cholesterol.
The risk evaluation: Normal (N): less than 200 mg/dl; Borderline (B): 200-239 mg/dl; High (H): more than or equal 240 mg/dl

Discussion

Among the 14 bacterial isolates tested, isolate no.8 isolated from raw cow milk has proven to be the most promising and active isolate to reduce cholesterol by (33.07%). Therefore, the current study results are compatible with many studies that proved the ability of various bacteria to lower cholesterol in a liquid media, such as Kulkarni *et al***,** who reported that bacterial isolated from raw cow milk demonstrated cholesterol degradation activity **(*Kulkarni et al., 2013*)**. In addition, it is consistent with a previous study that indicated the *Lactobacillius* isolates have the potential to diminish cholesterol **(*Khiralla, 2015*)**.

Isolate No 8 was characterized as the genus *Lactobacillus*, according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). Also, the isolate was identified based on the alignment of the 16S rRNA gene sequences available at GenBank. A BLAST (Basic Local Alignment Search Tool) search showed that *L. plantarum* MF1 showed 89.21 % approximation with *Lactobacillus plantarum* strain 1873 (accession number MT597698.). Isolate (No.8) was identified as a new strain of *L. plantarum* MF1 (accession No. MW242720.).

This work was expanded to include purification and characterization of cholesterol oxidase from *L. plantarum* MF1. The CHO enzyme was partially purified by precipitation with organic solvent (acetone) which agrees with **(*Bholay et al., 2013; Wali et al., 2019*)** who used acetone to precipitate the CHO enzyme produced by *Bacillus pumilus* W1,

Serratia marcescens W8, and *Bacillus licheniformis*, respectively. Precipitation was completed by adding acetone to broth in a 3:1 ratio, whereas **(*Bholay et al., 2013; Wali et al., 2019*)** precipitated the enzyme by adding acetone to broth in a 1:1 ratio.

The purification process for *L. plantarum* MF1 was carried out as follows: the rested precipitate was resuspended in the minimal amount of potassium phosphate buffer pH 7.0 and applied for Sephadex G100 column chromatography. The concentrated purified cholesterol oxidase was obtained by collecting and dialyzing the active fraction with a sharp peak on the fraction purification curve against the same buffer.

Furthermore, SDS-PAGE technique was used to determine the molecular weight of *L. plantarum* MF1 purified enzyme which exhibited as a single band with a molecular weight of 51 KDa. This result is compatible with the finding by ***Sanjeev et al* (2018)**, who determined the molecular weight of CHO enzyme of *Bacillus sonorensis* was 55 KDa.

The characterization of the purified enzyme was also investigated by testing the effect of various factors such as temperature, incubation period, substrate concentration, enzyme concentration, and some metallic ions on the activity of *L. plantarum* MF1cholesterol oxidase and comparing the results with the same enzyme purified from other references.

The maximum incubation temperature of CHO activity (2.8U/mL) was recorded at 30°C, while cholesterol oxidase showed significant activity at 20-50°C, with a maximum activity of 35°C **(*Lashkarian et al., 2010*)**.

The CHO activity of *L. plantarum* MF1 increased with incubation time increased until it reached 30 minutes (2.8U/mL), at which point it began to decrease slightly.

These results approved that the CHO activity increases exponentially with increased enzyme concentration. The increased enzyme activity of the tested CHO became non-linear until either a plateau was observed or further enzyme addition had no effect on CHO activity. This finding agrees with **(*Yehia et al., 2015*),** who announced that the rate of an enzyme reaction is typically proportional to the concentration of the enzyme**.**

The present work shows the correlation between substrate (cholesterol) concentration and cholesterol oxidase activity because the enzyme activity gradually increased as the substrate concentration increased. This result is consistent with **(*El-Naggar et al., 2017*)**, who illustrated that the influence of substrate concentration on the activity of *Streptomyces aegyptia* NEAE 102 cholesterol oxidase, as the enzyme activity increased with the substrate

concentration increased from 0.05 to 0.4 mM.

The current study explains how certain metallic ions act as activators or inhibitors of the activity of *L. plantarum* MF1 purified cholesterol oxidase.

The results indicated that $CuSO_4$ acted as activator for L. *plantarum* MF1 cholesterol oxidase, while $CaCl_2$, $MgSO_4$, $ZnSO_4$, and $MnCl_2$ inhibited cholesterol oxidase activity. These findings are consistent with those of **(*Rodrigues and Palani, 2016*)**, who discovered that Mn^+ and Zn^+ inhibited the *Enterobacter cloacae* cholesterol oxidase activity.

The results proved that the *L. plantarum* MF1 cholesterol oxidase was reached to the maximal activity at zero-day, and the enzyme retained around 57% of its initial activity after storage for 4 days at −20 °C. In contrast, *Streptomyces aegyptia* NEAE-102 cholesterol oxidase retained about 70% of its activity after storage at −20 °C for 8 months **(*El-Naggar et al., 2018*)**. This work extended to explore the impact of the purified CHO enzyme produced by *L. plantarum* MF1 on different serum cholesterol samples. The reduction ratio in all samples ranged from (18.1 – 29.0 percent), indicating a general decrease in the amount of cholesterol in all tested serum samples. This result is consistent with the result that the enzyme's effect on serum cholesterol was found to be 31.57% **(*Sanjeev et al., 2018*)**. Based on the findings of this research, we concluded that this enzyme is industrially important due to the high decreasing ratio of cholesterol in all samples, which is considered a good ratio for decreasing cholesterol *invitro* to be widely used for clinical purposes.

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الملخص العربى

استخلاص وتنقية وتوصيف إنزيم أوكسيديز الكوليسترول المُصنَّع حيوياً بواسطة بروبيوتيك *Lactiplantibacillus plantarum* MF1

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يهدف العمل الحالي إلى تنقية وتوصيف إنزيم الكوليسترول أوكسيديزالمُنْتج بواسطة بكتيريا بروبيوتيك المعزولة من حليب البقر الخام. و تم التعرف على العزلة البكتيرية المُنتجة على المستوى الجينى على *أنها* *Lactiplantibacillus plantarum* MF1 بواسطة 16S rRNA وتم تسجيلها في بنك الجينات برقم MW242720. تم تنقية إنزيم الكوليسترول أوكسيديز جزيئًا عن طريق الترسيب بواسطة الأسيتون تليها التنقية عن طريق السيفادكس G ١٠٠. ثم تم تعين الوزن الجزيئى لإنزيم الكوليسترول أوكسيديز باستخدام.(SDS-PAGE) وبدراسة الظروف المثلى اللازمة لأقصى نشاط لانزيم الكوليسترول أوكسيديز المستخلص من بكتيريا *Lactiplantibacillus plantarum* MF1 هى ان تكون مدة التفاعل ٣٠ دقيقة عند درجة حرارة ٣٠ درجة مئوية فى وجود ركيزة الإنزيم بتركيز ١٥٠ ميكروليتر و الإنزيم بتركيز ١٠٠ميكروليتر فى وسط التفاعل

فسرت الدراسة الحالية أن بعض الايونات المعدنية تعمل كمنشطات ومثبطات لنشاط إنزيم الكوليسترول أوكسيديز المنقى المُنتج من بكتيريا*Lactiplantibacillus plantarum* MF1 و أظهرت النتائج أن أعلى نشاط للإنزيم يكون فى وجود كبريتات النحاس بينما كانت كلوريد الكالسيوم وكبريتات الماغنسيوم وكبريتات الزنك وكلويد المنجنيز كمثبطات لنشاط الإنزيم. أوضحت النتائج أن إنزيم الكوليسترول أوكسيديز النقى يظل محتفظاً بـ ٥٧% من نشاطه الأصلى بعد أربعة أيام من الحفظ عند درجة حرارة - ٢٠مئوياً. وبتطبيق إنزيم الكوليسترول أوكسيديز المنقى على ٥٠عينة سيرم لدم إنسان مقاساً لها نسبة الكوليسترول مسبقاً وجد أن هناك إنخفاض ملحوظ فى نسبة الكوليسترول تصل الى ٢٩٪ بعد إضافة الإنزيم.