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EVALUATION OF STREPTOCOCCUS THERMOPHILUS EFFECTS ON LINOLEIC ACID (C18: 2) UNDER TEMPERATURE AND PH STRESS CONDITIONS

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ABSTRACT

Background and aim: Due to the beneficial physiological effects of conjugated linoleic acid (CLA), there has been a growing tendency to produce it as a functional lipid in recent years. In this study, optimization in lipid and linolenic acid production was investigated in as a Streptococcus thermophilus oleaginous Lactic Acid Bacteria.
Material and method: In present study, we added linoleic acid (concentration 5µg ml^{-1}) in the medium of Streptococcus thermophilus in M17 media, under different pH and temperature stresses, and after 24 h of incubation, produced CLA was estimated by Spectrophotometric and Gas chromatographic techniques.
Results: Our results indicated that Streptococcus thermophilus in M17 medium produces CLA in pH 7, but not in pH 4. This bacterium produces CLA at 37°C, but not at 45 °C.
Conclusion: The findings indicated that transformed Streptococcus thermophilus can be used for CLA production in biocatalytic processes.

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Introduction

Given that lipid is a large and heterogeneous collection of biological molecules, playing a major role in human's health, through energy storage for body and transmitting messages between cells and their environment. It's also used for structural combination of cell membrane. According to useful effects of multi-link unsaturated fatty acids, participating as a material for cell wall production, especially in brain and central nervons system and that, body is not able to produce it, it is received in body via daily nutrition [1 , 2].

Among the most important fatty acids, are Omega six (Linoleic acid) and Omega three (Linoleic acid). Naturally, Conjugated Linoleic acid (CLA) is a fatty acid belonging to the family of Omega six fatty acid and is a local isomer [3].

Isomer cis 9 / Trans 11 CLA in milk fat is active and dominant (75-90%).

The significance of this isomer was demonstrated in biomedical studies on lab animals after discovery of anti-cancer properties. Human can catch it from meat food sources and ruminant milk. In fact, CLA is a fatty acid with animal source , having useful properties on human's health. One way of its production is change of LA to CLA by means of Lactic acid bacteria, using desaturase enzyme in ruminant animal's body [4, 5]. Probiotics are non-pathogemc microorganisms, and are used greatly in drug and food industry. When entered in body in sufficient values, they affect the health positively.

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Since a huge part of probiotics belong to Lactic acid bacteria, for an optimized change of Linoleic acid to conjugated linoleic acid, two of most important lactic acid bacteria's named Lactobacillus acidophilus and streptococcus thermophiles both being probiotic were used in different stress conditions [6 , 7]. Knowing that different stress conditions can cause different behavior patter as of the molecules in bacteria, most of lactobacillus, lac tococcus, and strep tococcus strains are able to produce CLA from Linoleic acid in special environments or skimmed or full milk, but the production depends on culture medium totally [8,9]. Sucrose, lactose, fructose and in some cases sodium chloride imply negative effect on CLA production. Study to develop alternative methods seems necessary, and production of special and pure products is possible using them [11 , 10]. Therefore, the study aims to evaluate optimization of Linoleic acid production affected by PH and temperature by bacteria streptococcus thermopbilus.

Material And Methods

Isolation and culture of bacteria:

In the present study, the standard streptococcus thermophiles was produced using starter culture powder (yc - $38^{\circ}c$) from Hansen company. Some of Liofreeze powder was cultured in 2ml of liquid medium M17, for bacteria's passage.

Then, it was incubated at $37^{\circ}c$ for 24-48 hours and in aerobic conditions.

About 50 ML of M17 was transferred in to solid medium M17 and was cultured in stearic to isolate single colonies. The single colonies of medium were used for diagnostic tests of Gram stain. Catalase test and biochemical tests.

Directions to half McFarland suspension:

Using 0/05ml of Bac12.2H2O by a concentration of %1/175WN and adding in 9/95ml sulfuric acid %1 7/7 and fermentation of bacteria's pure colony into suspension solution of half McFarland, suspension including $1/5 \times 10^{8}$ CFU/ml was produced [7].

Evaluation of effect of streptococcus thermophiles on Linoleic fatty acid in temperature conditions:

First, 10ML of 5mg/ml stock of fatty acid was added to 990ML liquid medium M17. Then, it was uniformed by vertex. Next, an amount of 100ml of the concentration 50Mg/ml of fatty acid was added to 900Ml of bacterial suspension, it means half McFarland and then was uniformed thoroughly. Next, the prepared samples were transferred to Incubator at $37^{\circ}c$. In addition, another sample was produced similarly but was incubated at $45^{\circ}c$. To produce fatly acid of control group in liquid medium of M17, an amount of 100ML of concentration 50Mg/ml was added to 900ML sterile and liquid culture medium and then, it was uniformed. In following, the pipe was transferred to Incubator at $37^{\circ}c$. Similarly, fatty acid control was produced to be put in incubation at $45^{\circ}c$ [7].

Effects of streptococcus thermophiles on Linoleic fatty acid in PH conditions:

At first, 100ml of liquid medium M17 was prepared and Hydrochloric acid (12N) was added to it gradually, step by step for medium PH to be set on 4. Moreover, to produce 100ml liquid medium by a PH of 7, NaoH (6N) was used similarly.

Streptococcus thermophiles suspension was prepared based on half McFarland in M17 by 2 PH conditions of 4&7.

Furthermore, fatty acid of 50Mg/ml was produced in liquid medium M17 in above-mentioned PH. An amount of 100ML of Linoleic fatty acid in PH 4 and 7 was added and mixed with 900ML suspension of streptococcus thermophiles of half McFarland in liquid medium of M17 in the same PH, and the sample was transferred into incubator at the optimum temperature for bacteria growth. To control fatty acid, 100ML of it in M17 with PH of 4 and 7 was placed into 900ML of sterile and liquid medium M17 by PH 4 and 7 [7,8].

Linoleic fatty acid extraction:

After incubation of each sample in above-mentioned conditions, control and test samples were put in centrifuge. (5 min, $4^{\circ}c$, 3000gr) and then, the sediment was cleaned and the upper liquid was separated for fatty acids extraction.

Identification of Linoleic fatty acid combination:

The ability to change linoleic to conjugated linoleic acid was determined using bacteria, by spectrophotometry method.

In the method, Frist, streptococcus thermophiles was cultured in M17 agar, at $37^{\circ}c$ for 24 hours, and it was then put in centrifuge (5min, $4^{\circ}c$ and 7500g). Next, 1/5ml of upper liquid was mixed with 3ml Isopropanol and was in vertex process for 1 minute.

An amount of 2/5ml hexane was added to sample and then mixed and put in centrifuge (5 min, $4^{\circ}c$, 2000g). The separated upper liquid was added into Quartz Cuvette and examined in spectrophotometer (ware length of 233 Nm).

Fatty acid Analysis:

The method Falch was used in the study to be able to extract fat. For this purpose, the samples were mixed with 10 ml chloroform: Methanol 2:1 and after that, centrifuge was don on sample (1 min, 2575gr), the upper phase was thrown away. Finally, 1 ml Hexane was added to tube, after 30 seconds vertex was don and sample was moved to a 1/5 ml micro tube. The stock 3- decanoic acid of 5 mg/ml was wed as internal standard. First, 25 Ml c13 fatly acid (with a concentration of 125 Mg/ml) was added to 475Ml sample as an internal standard. After verlex , the sample containing c13 was dried by Nitrogen gas, again. Then, 200Ml Hexane was added to it and vertex was done once more. Next, 100 Ml NaoH of Methanol kind was added and after another vertex, the samples were incubated for 30 mins at $37^{\circ}c$. Afterwards, 300ML BF3 was mixed with samples and after another vertex, the samples were incubated for 30 mins at $37^{\circ}c$. Hereafter, they were under vertex by mixing of 300ML Hexane, centrifuge was done (1 min, 2575 g) and then the upper liquid was separated. It is worth noting

that before injection of samples, standards of CLA, LA and also C13, as internal standards should be considered. The gas chromatography device, used in
Done to compare bacteria test and control groups. The results were compared by (P < 0/05).

Results

The results from bacteria incubation at 37°c for 24 hours, approved that, the single colonies were separated following bacteria culture in M17. Micro and clear colonies were grown up well in agar M17 and it would be the first sign of the possibility of streptococcus thermophiles bacteria existence.
Afterwards, bacteria strains were approved through biochemical and morphological tests. Absorption produced by linked CLA was measured in wavelength 233 Nm. Through the device spectrophotometer. In addition, a standard curve was drawn using various concentrations of 0-10-20-30 Mg/ML from CLA, by spectrophotometer device and it was compared with the results as in table 1. The results from spectrophotometry and light absorbance of surface liquid from streptococcus thermophiles culture is shown in diagram 1, in liquid medium M17 in wavelength of 233 NM. CLA absorbance in wavelength of 233 Nm by streptococcus thermophiles in M17 at 37°c showed no significant difference comparing with control group (p=0/69): however, a slight difference was observed compared with control group at 45°c (p=0/12), its results lacking a meaningful statistics. The results coming from gas chromatography analysis showed that at first, the standards Linoleic acid (cis 9-cis isomer 12), conjugated linoleic acid (Trans – 11 cis 9 – isomer) and c13 diagrams were drawn, and as it can be observed in diagram 2, the inhibition time of c13, Linoleic acid and conjugated linoleic acid was in a time limit of 10/3-10/4 , 15/6 and 17/4-17/5 miss, respectively. Streptococcus thermophiles analysis in M17 under temperature and PH stress, comparing with control samples, demonstrated that CLA was produced by a surface under diagram 92/70%. In M17, under temperature stress of 37°c and Linoleic acid reached 7/29% under the curve, but No CLA was produced at temperature stress of 45°c (diagram 3). Regarding PH stress, CLA was produced from streptococcus thermophiles sample in M17, under stress of PH =7, by 89/70%. Under the curve and the percentage of surface under Linoleic acid reached 10/29%, but No CLA was created in PH=4.

Discussion

In the present study, streptococcus thermophiles was incubated for 24 hours in separate PH and temperature stress, after adding linoleic acid to medium (concentration of 5Mg/ml).
Then it was analyzed by gas chromatography Technique and Spectrophotometry. In the present study, the ability to change linoleic acid to conjugated linoleic acid was determined using bacteria by spectrophotometry method. The overall results showed that the light absorbance of CLA showed no significant difference in wavelength of 233 Nm using the bacteria streptococcus thermophiles in M17 at 37°c , compared with control group; while, a slight difference was observed at 45°c compared with control group. In the analysis, components in M17, seemed to be bothersome, due to medium components containing peptone, and its derivatives, meat extract, and yeast extract, having so much fatty acid, and as a result, in addition to light absorbance of CLA, light absorbance of fatty acid of many other types happened in wavelength of 233 Nm, meaning that no significant result was made in the medium especially for CLA light absorbance, and as it is observed, there was no significant difference between acid control and test sample, due to high light absorbance in medium components. The related results to gas chromatography analysis shows that temperature stress of 37°c for streptococcus thermophiles was effective in CLA production, despite the fact that no CLA was produced at 45°c. The result shows that the temperature 37°c is a more suitable degree for enzyme's production including isomer from linoleic acid, that is producing CLA. It was observed through an analysis of that sample of streptococcus thermophiles in M17 that an amount of 54/34% of CLA was produced in p4=7, considering that the mentioned medium has a PH of 7/2. Due to closeness of PH=7 to the medium PH, no stress was imposed on bacteria and it was grown up normally in its suitable medium; while, no CLA was given out in PH=4. Against the study of Fozo in 2004, showing that growth of organism in acidic environment will cause an increase in unsaturated fatty acid; if changes to increase unsaturated fatty acid is limited by adding fatty acid biosynthesis inhibitor, organism will show more sensitivity to acid [16].
At the moment, Linoleat isomerase exists in most of bacteria, so that, CLA production has been reported in so many bacteria [7,17]. But, it is proved generally that LABS are able to change unsaturated fatty acid to Hydroxides of fatty acid, since the bacteria used in the present study is one of lactic acid bacteria's and along with present studies, CLA was produced using LA substrate by desaturase and linoleat isomerase activities existed in Lactic acid bacterias [18]. Moreover, according to the results from C.P.Van Nieuwenhove et al studies in 2006, it is observed that production ability of CLA from LA was the highest using lactobacillus casein, lactobacillus Rhamnosus and Bifidobactrium and streptococcus thermophiles [9]. As it is observed, bacteria's didn't produce conjugated linoleic acid in Acidic PH and at 45°c. The results are in conformity with the studies by kabara in 1993. It was shown in studies that PH implies a serious a serious effect on fatty acid's performance, and less PH leads to higher rate of microbial death [19,20] suggested that after acid stress, a decrease in linoleic unsaturated fatty acid concentration could be a part of survival mechanism for removing proton concentration to be increased in cell during acid stress, that is conformed with the results of present study [20]. Rodriguez-Aleala et al. (2011) showed that probiotics produce CLA1 and then CLA2 mostly, in the process of CLA production from LA [2]. The process of CLA

production during fermentation is affected by many factors including bacteria strains, number of cells, the used substrate concentration and incubation time. CLA production depends on LA concentration in the medium at the first phase and then bacteria species [12,22].

It is determined in the present study that streptococcus thermophiles bacteria is able to produce CLA potentially as a probiotic bacteria to increase woeful compounds. Some species of streptococcus produce meaningful amounts of fatty acid in special conditions, during lipolytic process.

Conclusion:

Based on the results, transgenic streptococcus thermophiles cells can be used properly in CLA production by bio catalase processes.

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