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RESEARCH ARTICLE

Enhanced expression of lactate dehydrogenase genes in *Lactobacillus* using capsaicin enzyme

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Abstract Lactic acid is the most important organic acid produced by Lactic acid bacteria from sour milk. Lactic acid has wide application in pharmaceutical, chemical and food industries and also in the preparation of biodegradable polymers for medical uses such as surgical sutures, prostheses and controlled drug delivery systems. Lactic acid exists in two optically active stereo-isomers, the L(+) and the D(-). The L (+) lactic acid form is being preferred since the D(-) form is harmful in humans. Lactic acid can be manufactured either by chemical synthesis or by microbial fermentations. But microbial fermentation has significant advantage over the chemical system in utilizing cheap raw materials as substrates in L(+) lactate production. During microbial fermentation, to increase industrial productivity of lactic acid, genetic modification of starter culture is usually practised. However, Capsaicin (8-methyl-N-vanillyl-6-nonemide), an active ingredient of chili peppers(*Capsicum annum*), is said to increase the metabolic activity of *Lactobacillus* by triggering lactate dehydrogenase (LDH) gene responsible for conversion of pyruvate to lactate. Therefore the effect of capsaicin enzyme in enhancing ldh gene expression in *Lactobacillus* by continuous fermentation was studied thereby achieving economic feasibility.

Key words: L(+) Lactate, Lactate dehydrogenase, ldh gene, Capsaicin, Lactobacillus, microbial fermentation

Introduction

Lactic acid produced by lactic acid bacteria (LAB), is used in pharmaceutical, chemical and food industries primarily as an acidulant and preservative agent (Roukas and Kotzekidou 1998). Lactic acid has long been used in pharmaceutical formulations, mainly in topical ointments, lotions, and parenteral solutions. It also finds applications in preparation of biodegradable polymers for medical uses such as surgical sutures, prostheses and controlled drug delivery systems (Wee *et al.* 2006). Lactic acid is used in disinfection and packaging of carcasses, particularly those of poultry and fish, where the addition of aqueous solution during processing increased shelf life and reduced microbial spoilage (Datta *et al*. 1995, Naveena 2004). More than 50% of lactic acid produced is used as emulsifying agent in bakery products (Datta *et al*.1995, Litchfield 1996). The water retaining capacity of lactic acid makes it suitable for use as a moisturizer in cosmetic formulations. Ethyl lactate is the active ingredient in many anti-acne preparations (Wee *et al.* 2006).Lactic acid exists in two optically active stereo-isomers, the L (+) and the D (-). Elevated levels of D (-) lactic acid is harmful to humans, as human have only L- Lactate dehydrogenase that metabolizes L (+) lactic acid (Akerberg 1998, Hofvendahl 2000).

Lactic acid can be manufactured either by chemical synthesis or by microbial fermentations. Chemical synthesis result in racemic DL-lactic acid whereas stereospecific [L(+), D(-) and DL mixture] form is produced by fermentation using specific microbial strain (Datta *et al.*1995, Litchfield 1996). Thekey enzyme in homolactic fermentation is lactate dehydrogenase (LDH), which converts pyruvate to lactate and regenerates NAD+ for the continuation of glycolysis (Neves *et al*. 2000, 2005). LDH activity is widely spread in all domains of life and in certain bacteria LDH activity is encoded by multiple ldh genes (Neves 2000, Gaspar, 2007).Bacterial fermentation reduces the pH of milk by conversion of milk sugar lactose into lactic

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acid (Lee and Lucey 2010, Lucey 2004). Traditionally in India; stems of chili peppers (*Capsicum annum*) are used for starter preparation (Lilija 1998). In general household practice, curdling is known to occur faster in the presence of chili particularly in the colder regions or during winters or during shortage of starter cultures (Katz 2011). The studies showed that there is an enhancement of L-lactate production in the presence of pure capsaicin enzyme which is the major component of *Capsicum annum*(Sharma *et al*. 2013). Recently Maitham *et al*. 2021 has reported the role of lactate dehydrogenase in cancer pathogenesis. The present study was designed to establish the effect of the aqueous extract of green chili (*Capsicum annum*) which contains the active component capsaicin in enhancement of lactate production.

Aim

To enhance the lactic acid productivity by *Lactobacillus lactis* using capsaicin enzyme extracted from *Capsicum annuum*.

Objectives

To isolate *Lactobacillus* from the serially diluted curd sample.
To detect Lactate dehydrogenase (LDH) gene present in the isolates by PolymearseChain Reaction.
To extract capsaicin enzyme from the ripe fruits of *Capsicum annuum*.
To demonstrate continuous batch fermentation with capsaicin enzyme for enhanced expression of ldh gene.
To analyze the lactic acid content using titration methods.
To determine the cell counts frequently to check the correlation between the cell count and amount of lactic acid produced.

Methodology

Starter Culture

Lactobacillus lactis, the facultative anaerobes of Lactic Acid Bacteria (LAB) group, used in this study were isolated from curd sample by serial dilution and spread plate technique on deMan, Rogosa and Sharpe (MRS) agar medium.The medium has protease peptone, beef extract, yeast extract and dextrose as nutrient base and selective agents such as sodium acetate and ammonium citrate to inhibit normal flora and the presence of polysorbate, magnesium and fatty acid, favarouble for the growth of *Lactobacillus*, thus making it a selective medium for isolation of these bacteria from the samples. The pH of the medium was adjusted as 6.4 to further promote the growth of *Lactobacillus*. The pure culture thus obtained was maintained by regular sub culturing in MRS medium.(Vishnu 2000, 2002).

DNA Extraction and PCR amplification of ldh genes

For the extraction of genomic DNA by boiling method, the bacterial pellets were briefly suspended in 200µL of TE buffer (Tris-HCL [10mM]: EDTA [1 mM]) and subjected to 15 minutes of boiling. The microfuge tubes were placed in an ice bath for 15 minutes and then centrifuged for 5 minutes at 14,000 rpm at room temperature. The supernatant containing DNA (100µL) was transferred to another clean tube and stored at -20˚C.The extracted DNA was subjected to PCR to amplify the ldh gene using the forward (5'ACACGCCCATCCGAGCAGG3') and reverse primers(5'GCACAGGCACCAATTCCATAAAA C3') for ldhgene. PCR was carried out in a volume of 50µl containing 25µL master mix from commercial kit (Aqua Red Taq 2x Master mix); 2µl forward and reverse ldh primers(obtained from Lexus scientific, Chennai) and 10µl of isolated DNA and made up to 50µL using deionized water. The amplification reactions were performed in a PCR thermo cycler and the temperature profile was as follows: initial denaturation step (2 min at 94˚C) followed by 30 cycles of amplification (denaturation at 94˚C for 30seconds, annealing at 58˚C for 30seconds and elongation at 72˚C for 30seconds). Following amplification, 10µl of the PCR mixture was analyzed by 1.5% agarose gel electrophoresis using a 100bp DNA marker.

Extraction of capsaicin enzyme from *Capsicum annuum*

Capsicum annuum unripe whole fruits, harvested on the 40th day (G-4 green chilli variety, the whole fruit which were 8.5 cms length, meant to produce high quantity of capsaicin, i.e., nearly 0.52mg/

gram of chilli was used for the study) were surface sterilized by immersing in sodium hypochlorite solution for 2 to 3 min, rinsing thoroughly with sterile water for3 to 4 times and dried in laminar air flow hood. The appropriate amount of 400mg of chili was weighed, mashed, mixed thoroughly with sterile water by vortexing. The extract was then filter sterilized and stock solutions were prepared.(Though literature survey showed different concentrations of capsaicin produced in different parts of the fruits as reported by Veronika *et al.* in 2007, whole unripe green chilli fruits were used for the study since the aim was to trigger ldh gene using capsaicin enzyme and not the concentration).

Fermentation process for Lactate production

Continuous batch fermentation was carried out for 5 consecutive days in an Erlenmeyer flask, containing 150ml of production medium (Nutrient broth) with a loop full of maintained pure culture at 37˚C. This was made as 3 sets A, B, C, respectively.
A- Production medium without capsaicin enzyme (control) sterilized at 121˚C for 15 min.
B- Production medium with capsaicin (test) and subjected to sterilization at 121˚C for 15 min.
C- Production medium sterilized and unsterile capsaicin enzyme added at room temperature (test).

Determination of cell count

Incubated cultures were monitored for rate of cell growth using UV-visible spectrophotometric analysis at OD620. The growth rates were calculated everyday through linear regression with a wavelength of 620nm against the time duration.

Determination of pH

The pH of the fermenting substrates was measured with the electrode of a pH meter standardized with appropriate buffer.

Estimation of lactic acid produced

The amount of lactic acid produced during the fermentation was determined daily for 5 days by the standard titration procedure for total titratable acidity (TTA) according to Association of Official Analytical Chemists (A.O.A.C), 1975,1990, Arlington, VA. Lactic acid content determination was done by titrating 25ml of supernatant fluid of the substrates on addition of 3 drops phenolphthalein as indicator, 0.1M sodium hydroxide (NaOH) was slowly added from a burette into the samples until a pink color appeared. Each ml of 0.1M NaOH is equivalent to 90.08mg of lactic acid.

$$\text{Total titratable acidity of lactic acid (mg/ml)} = \frac{\text{mlNaOH x N NaOH xM.E}}{\text{volume of sample}}$$

Where, ml NaOH = Volume of NaOH used,
N NaOH = Molarity of NaOH used,
M.E =Equivalent factor = 90.08mg.

Results

Starter culture

Pure cultures of *Lactobacillus* were isolated from the serially diluted curd samples through spread plate technique on MRS agar plates as shown in fig. 1. The preliminary and biochemical tests were carried out for the confirmation and identification purposes. The identified organisms were sub cultured in MRS broth medium for further analysis.

DNA extraction and PCR amplification of ldh genes

The extracted DNA from the *Lactobacillus* culture was subjected to Polymerase chain reaction for amplification of ldh gene**. (**Figure 2).

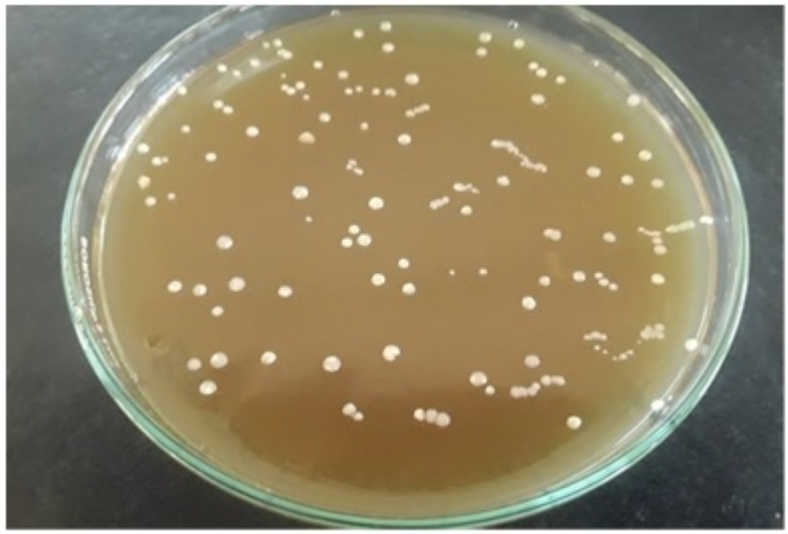
Fermentation process for Lactate production

Three sets of production media were prepared as mentioned in methodology with Nutrient broth alone (plain, A), Nutrient broth with sterilized capsaicin enzyme (after autoclaving, B), and Nutrient broth with unsterile capsaicin enzyme (before autoclaving, C), inoculated and incubated. Following incubation, the production media observed for turbidity. **(**Figure 3).

Determination of cell count

The cell count was calculated by UV-Visible spectrophotometric analyses, where the amount of light transmitted was obtained at 620nm wavelength. This was carried out for 5 consecutive

Figure 1: *Lactobacillus* from curd sample on MRS agar medium.



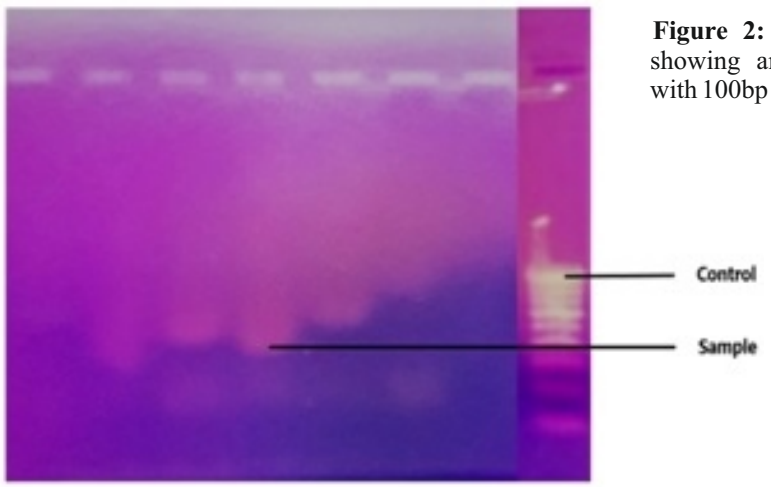


Figure 2: Agarose gel Electrophoresis showing amplified ldh gene compared with 100bp DNA ladder

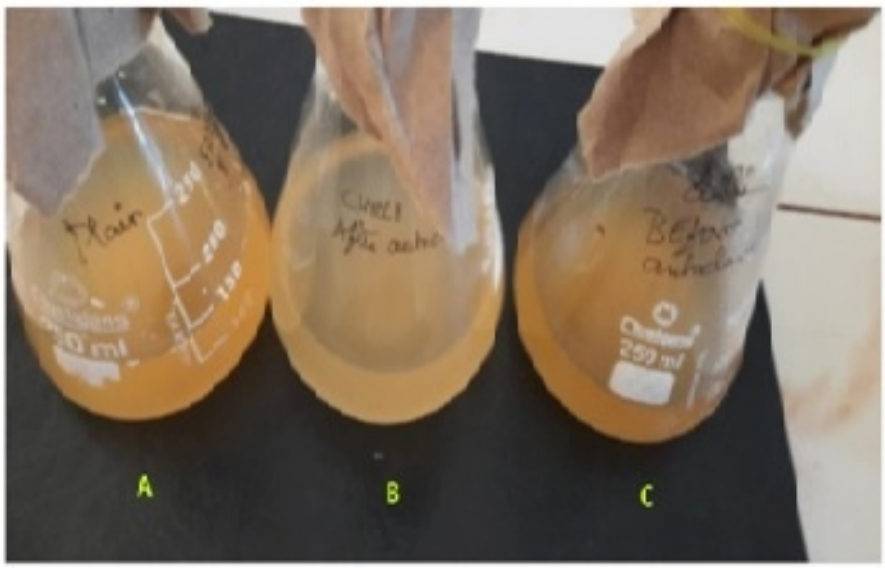


Figure3: Production media showing turbidity

Table 1: Cell count obtained at 620nm wavelength

DAYS	CELL COUNT		
	PLAIN	AFTER	BEFORE
DAY 1	2.8	2.6	3
DAY 2	4.7	6	5.1
DAY 3	3.9	12.5	16.8
DAY 4	10	15.2	14.7
DAY 5	12.5	17.4	13.9

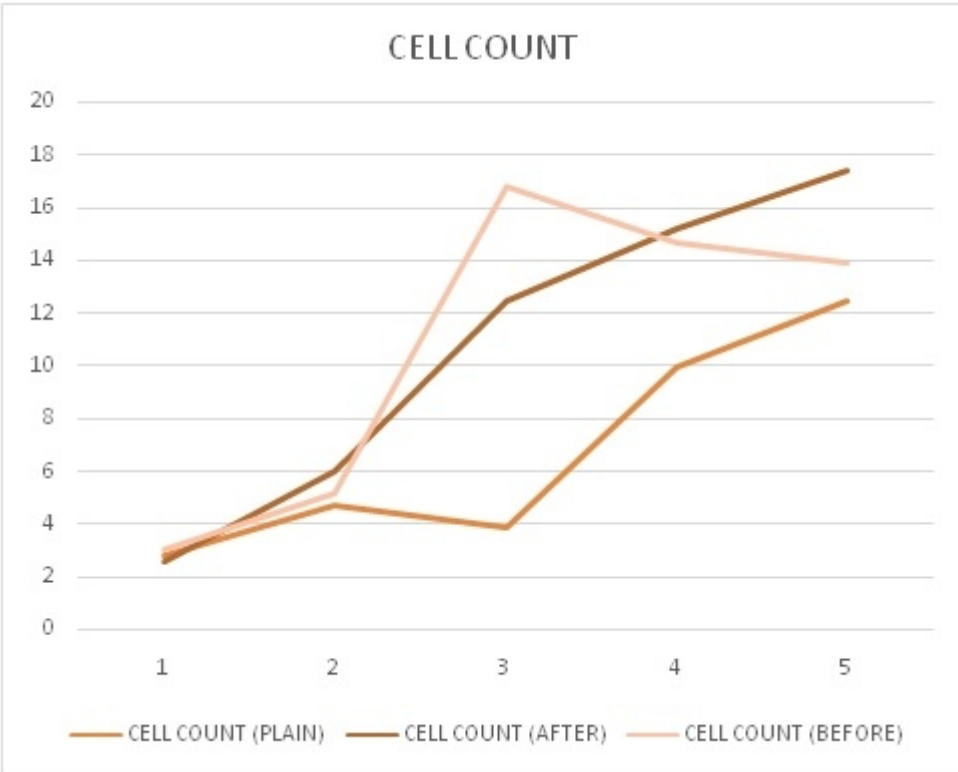


Figure 4: Cell count obtained at 620nm wavelength

days and the values are recorded as shown in table 1 and figure 4.

Determination of pH

The pH values for three sets of media were determined after fermentation and were found to be as follows

A- Production medium without capsaicin enzyme (control) = 4.0

B- Production medium with capsaicin (test)=3.6

C- Production medium sterilized and unsterile capsaicin enzyme added at room temperature (test)=3.8

(the initial pH in all the media was 6.4)

This showed that the supplementation of media with capsaicin enzyme decreased the pH there by increasing the productivity of lactate.

Estimation of lactic acid produced

The total titratable acidity was determined for the amount of lactic acid produced by standard titration method and estimated using the formula as mentioned in the methodology. The results were tabulated as shown in the table 3 and figure 5.

Table 2. Lactic production estimated in mg/ml

DAYS	LACTIC ACID CONTENT (mg/ml)		
	PLAIN	AFTER	BEFORE
DAY 1	2.08	1.87	2.23
DAY 2	2.05	2.55	3.24
DAY 3	1.94	2.41	2.34
DAY 4	1.90	2.52	2.55
DAY 5	1.80	2.70	2.70

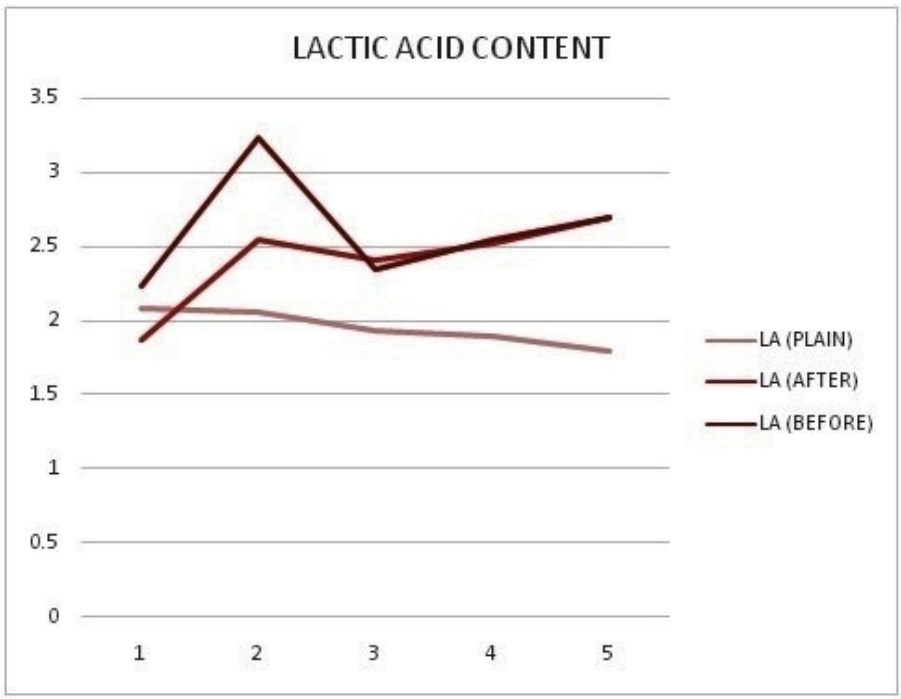


Figure 5: Lactic production estimated in mg/ml

Discussion

The present study was aimed at finding the effect of capsaicin in enhancing the expression of ldh gene in *Lactobacillus*. Usually such enhancements were achieved by genetic modifications or strain improvement techniques, which may require adequate time and investment. Instead of such expensive procedures, using the alternative plant extracts resulted in increased productivity of lactic acid. Also the optically pure stereo isomers of lactic acid can be obtained.Lactic acid bacteria are characterized by the production of lactate as the main fermentation product where the carbohydrate substrates are catabolized to pyruvate through glycolysis pathway. Further the fate of pyruvate is decided by the enzymes produced on expression of various genes. Lactate dehydrogenase is the enzyme responsible for the conversion of pyruvate into lactate. Lactic acid bacteria being fastidious microorganism requires multiple amino acids and vitamins for growth, hence the MRS medium is used for the isolation and maintenance of *Lactobacillus lactis*. Lactic Acid Bacteria (LAB) consists of about 20 genera of which *Lactobacillus*is largest (Axelsson 2004). Therefore in the current work, Lactobacillus was isolated from curd samples on MRS medium.

The genomic DNA was extracted from the isolates

through the standard boiling method since it is as efficient as the commercial kit, but much less costly and laborious (Rubeiro *et al*. 2016). Further the extracted DNA was subjected to PCR technique with forward and reverse primers of ldh gene. This was carried out for the confirmation of presence of the gene. Similarly in the present study, the presence of ldh gene was confirmed by extraction of genomic DNA and amplification of the genes by PCR method.

The aqueous extracts of capsaicin enzyme from *Capsicum annuum*fruit was obtained by pulverizing and mixing thoroughly with sterile water as the highest concentrations were found to in the ovary and in the lower flesh of the fruit. **It was found that the Takanotsume variety produced 227mg of capsaicin in the ovary, 122mg in the bottom flesh , 21mg in the upper flesh and 6.2mg in the seed / 100 grams of fresh weight of the fruit. This is in contrast to CeceiFellallo variety which produced 19mg, 2.7mg, 1.8mg and 2.2mg respectively/100grams of the fresh fruits.** The capsicum fruit was surface sterilized using 2% sodium hypochlorite and the extract was filter sterilized to prevent the interference of contaminations (Veronika *et al.* 2007, Sharma *et al*. 2013).The capsaicin enzyme was capable of influencing the expression of the ldhgenethus enhancing the production of lactate dehydrogenase which intensifies the conversion of pyruvate into lactate (Sharma *et al*. 2013). Also the enhanced effect of capsaicin enzyme on L-lactate production at various temperatures was more profound.

Four types of chili (*Capsicum annuum*) extracts, categorized according to color; green and red, and size; small and large were studied in Hep-G2 cells. Red small (RS) chili had an LC50 value of 0.378 ± 0.029 compared to green big (GB) 1.034 ± 0.061 and green small (GS) 1.070 ± 0.21 mg/mL. Red big (RB) was not cytotoxic. Capsaicin content was highest in RS and produced a greater percentage sub-G1 cells (6.47 ± 1.8%) after 24 h compared to GS (2.96 ± 1.3%) and control (1.29 ± 0.8%) cells. G2/M phase was reduced by GS compared to RS and control cells. RS at the LC50 concentration contained 1.6 times the amount of pure capsaicin LC50 to achieve the same effect of capsaicin alone. GS and GB capsaicin content at the LC50 value was lower (0.2 and 0.66, respectively) compared to the amount of capsaicin to achieve a similar reduction in cell growth.(David *et al*. 2014). Bindu *et al*. (2009) had reported the therapeutic and adverse effects of capsaicin in a dose-dependent manner. They studied 16 samples of *Capsicum* fruits grown at different altitudes in Nepal and determined their capsaicin content by high-performance liquid chromatography as the prerequisite for optimizing the formulation based on *Capsicum* fruit as a crude drug. The capsaicin content was found to range from 2.19 to 19.73 mg per g of dry weight of *Capsicum* fruits. Capsaicin content in pericarp was found to be higher than in seeds. No correlation was found between the shape or size of the fruits and its capsaicin content. In this study, the production medium was supplemented with capsaicin enzyme at two different temperature conditions, which revealed the differences in lactate production.However only ripe whole fruits alone were used for the study, since the idea was to trigger ldh gene expression using capsaicin enzyme produced from *Capsicum annum*and not to find the concentration of capcaisin in different parts of the fruit or the age of the fruit.

The cell count and the lactic acid productivity was analysed by standard method suggested by Association of Official Analytical Chemists (A.O.A.C), 1975 & 1990, Arlington, VA. and the correlation between cell count of LAB bacteria and lactate production was confirmed. Also Sharon *et al*., (2016) has analysed that capsaicin, an active ingredient in chillies, has improved control of insulin secretion and hence can be useful in the treatment of obesity, diabetes and heart disease. Thus the present study on capsaicin and its enhanced production by triggering ldh gene has future medical implications.

Conclusion

To enhance the lactic acid productivity by *Lactobacillus* spp. using capsaicin enzyme extracted from *Capsicum annuum*, *Lactobacillus* spp. was isolated from the serially diluted curd sample and was confirmed by the preliminary and biochemical tests.The isolates were screened for the presence of ldh gene by PCR technique and the amplified gene products confirmed by Agarose gel electrophoresis. The aqueous extract of capsaicin enzyme from *Capsicum annuum* fruit was obtained by pulverizing the fruits and the stocks were prepared after filter sterilization. The continuous

fermentation process was carried out using production media in three sets for 5 days as follows- without capsaicin enzyme (first set), with sterilized capsaicin enzyme (second set) and with unsterile capsaicin enzyme (third set). The productivity of lactate in different sets were compared following fermentation and was found to be more in the medium with capsaicin enzyme. Thus the enhance productivity of lactate using capsaicin enzyme was confirmed.

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