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CHOLESTEROL BIOTRANSFORMATION TO CHOLESTA-4, 6-DIEN-3-OL AND EFFECT OF ASSIMILATION ON ADHESION PROPERTIES OF LACTOBACILLUS HELVETICUS CD6

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ABSTRACT

Cholesterol biotransformation by *Lactobacillus helveticus* CD6 was observed in minimal medium supplemented with 3 mM cholesterol when grown for 120 h at 37 °C. Its gas chromatography-mass spectrometry (GC-MS) showed production of cholesta-4, 6-dien-3-ol and cholest-5-en-3-ol (3.beta) with 12 U/mg cholesterol oxidase-like enzyme activity. The cholesterol assimilation was evaluated at varied concentrations of bile salt in MRS medium. The cell survival and cholesterol assimilation was found to be adversely affected in presence of bile salt. Microscopic studies revealed changed cell morphology when grown with cholesterol. The cell adhesion properties like autoaggregation, microbial adhesion to solvents where found to be affected by cholesterol. The 7.49 % cell adhesion to ethyl acetate indicates the decrease in electron accepting properties of cell surface, while 9 % decrease in xylene adhesion and 13 % decrease in autoaggregation was observed which would be helpful in cholesterol lowering when supplemented in the form of probiotic preparation.

Keywords: Cholesta-4, 6-dien-3-ol, Cholesterol, Cholesterol oxidase-like enzyme, *Lactobacillus helveticus*

INTRODUCTION

Cholesterol is an important basic building block for body tissues and human metabolism. It is essentially required for the human body to insulate nerves, make cell membranes; where it acts as an important lipid to produce certain hormones (**Ma, 2006**). But, high cholesterol in serum is a leading risk factor for human cardiovascular disease (CVD) such as coronary heart disease and stroke (**Tabas, 2002**). CVD is the most important cause of death in the westernized countries and it is strongly associated with hypercholesterolemia (**Ahire *et al.*, 2012; Hansson, 2005**). The cholesterol levels in blood and the consumption of cholesterol rich diets are closely related to each other. Medical profession prompted to advise such patients to reduce their blood cholesterol levels to control coronary heart diseases (**Chauhan, 2007**). WHO predicts that by the year 2030, up to 40% of all deaths will be related to CVD, although; natural and drug-based therapy is currently being used to treat this condition (**Levine *et al.*, 1995; Thompson and Ernst, 2003**).

Lactic acid bacteria (LAB) are important microorganisms in the intestines of healthy humans. They have been associated with several probiotic effects in humans and animals such as overcoming lactose intolerance, increased immune responses, lowered blood cholesterol, alleviation of some diarrhoea, and prevention of cancer (**Fernandes *et al.*, 1987**). Recently, the consumption of LAB has been tested as an effective, safe alternative approach to manage cholesterol related problems (**Kumar *et al.*, 2011**), although, this needs huge validation studies in animal and human models. Several mechanisms have been proposed for cholesterol removal, including assimilation, cell wall binding, microbial transformation, enzymatic deconjugation, assimilation leading to intracellular degradation (**Gilliland *et al.*, 1985; Nakajima *et al.*, 1992; Freier *et al.*, 1994; De Smet *et al.*, 1994; Ahire *et al.*, 2012**). In this study, strain *Lactobacillus helveticus* was found to assimilate cholesterol in presence of bile salt and decreased hydrophobicity on assimilation demonstrates possible cholesterol lowering in diet through passage of cells with stool.

Adhesion to intestinal epithelial cells is an important prerequisite for colonization of probiotic strains in the gastrointestinal tract, preventing their immediate elimination by peristalsis and providing a competitive advantage in this ecosystem (**Alander *et al.*, 1997**). The probiotic properties like microbial adhesion to solvents and autoaggregation are non-specific physical interactions between two surfaces which directly reflect adhesion ability to enterocytic cellular lines (**Perez *et al.*, 1998; Del Re *et al.*, 2000**). These properties vary from organism to organism (**Ahire *et al.*, 2013, 2011a, 2011b**).

The cholesterol biotransformation by food-grade, non-toxic bacteria is less frequently reported (**Kumar *et al.*, 2001; Lye *et al.*, 2010**). However, **Ahire *et al.* (2012)** demonstrated biotransformation of cholesterol to androsta-1, 4-diene-3, 17-dione (ADD) and androst-4-ene-3, 17 dione (AD) in the presence of nucleus cleavage inhibitor 2,2′-dipyridyl by cholesterol oxidase-like enzyme. In this study, the transformation capabilities of strain were confirmed by detecting cholesterol oxidase-like activity and oxidized product cholesta-4, 6-dien-3-ol in minimal medium lacking nucleus cleavage inhibitor.

The aim of present study was to evaluate *Lactobacillus helveticus* for cholesterol biotransformation in absence of nucleus cleavage inhibitor and study its ability to assimilate cholesterol in the presence of bile salt followed by adhesion properties.

MATERIAL AND METHODS

Bacterial strain and cultivation

Potential probiotic strain CD6, isolated from conventionally fermented milk, was identified as *Lactobacillus helveticus*, as reported by **Ahire *et al.* (2013)**. It was cultured in de Man Rogosa Sharpe (MRS) broth (pH 6.5) for 18 h at 37 °C prior to use. Stock cultures were maintained at −80 °C, using 20 % glycerol as a cryoprotectant.

Cholesterol biotransformation

One mL overnight grown culture was cultivated in 100 mL tryptone soytone broth (TSB) (1.7 g tryptone; 0.3 g soytone; 0.3 g dextrose; 0.5 g NaCl; 0.25 g K_2HPO_4, pH 7.0) for 24 h at 37 °C. Transformation was carried out by inoculating 1 mL of 18 h old culture (6.9 $\log_{10}$ CFU/mL) from TSB into 100 mL transformation medium (1 g NH_4NO_3; 0.25 g $MgSO_4$.$7H_2O$; 0.25 g K_2HPO_4; 0.001 g $FeSO_4$; 0.005 g NaCl; 2 mL Tween 80; and 1 g cholesterol in 1L deionised water, pH 7.2) at 37 °C under microaerophilic conditions. Cholesterol transformation was analysed at every 24 h by thin layer chromatography (TLC) and enzyme assay. The sample showing highest enzyme activity was extracted by ethyl acetate and analysed on GC-MS.

Enzyme preparation

Cells in transformation medium were separated by centrifugation at 6,000 × *g* for 10 min (4 °C). The resulting supernatant was collected and used as extracellular enzyme, while separated cells were washed twice with phosphate buffered saline

(PBS; pH 7.3) followed by deionised water. After washing, cells were resuspended in 5 mL deionised water for ultrasonic disruption. Sonication was performed at 130 W with 30 % amplitude for 5 min in 50 s on/10 s off cycle at 0 °C using sonicator (VCX 130, Sonics Vibra cell, USA). Cell debris was removed by centrifugation at 6,000 × *g* for 10 min and resulting supernatant was used as intracellular enzyme (**Ahire *et al.*, 2012**).

Determination of cholesterol oxidase activity

Cholesterol oxidation activity was assayed by measuring generated H_2O_2 accompanying the oxidation of cholesterol (**Allain *et al.*, 1974; Ahire *et al.*, 2012**). In brief, 125 µL enzyme was incubated with 2.5 mL reaction mixture (64 mM sodium cholate, 0.34 % (v/v) Triton X-100, 1.4 mM 4-aminoantipyrine, 21 mM phenol, 0.9 mM cholesterol, 1.5 U peroxidise /mL) at 37 °C for 5 min followed by the measurement of increase in absorbance at 500 nm. The 1 U of enzyme activity was defined as the formation of 1 µM H_2O_2/min.

Analytical methods

Thin layer chromatography (TLC)

It was performed on silica gel 60 F254 (20 × 20 cm) plate (Merck, Germany) with hexane: ethyl acetate (7:3, v/v) and visualized by spraying the plates with a mixture of methanol: sulphuric acid (6:1, v/v) followed by heating in an oven at 105 °C for 3 min until the development of color.

Gas chromatography-mass spectrometry (GC-MS)

It was performed on a QP 2010 system (Shimadzu) interfaced to a mass detector and equipped with an Ultra-2 cross-linked capillary column (5 % phenyl–95 % methylpolysiloxane bonded phase; 25 m × 0.20 mm, 0.11 µm film thickness) (**Ahire *et al.*, 2012**). The injector, interface and ion source were at 260, 300 and 230 °C, respectively. Helium was used as the carrier gas at 0.5 mL/min. Samples were introduced in split-injection mode (10:1) and the oven was set initially at 240 °C (1 min) and then programmed to rise up to 300 °C (10 min) at a rate of 20 °C/min.

Cholesterol assimilation in presence of bile

It was studied as per the method described by **Gilliland *et al.* (1985)** with certain modifications. Freshly prepared MRS broth was supplemented with 0.0, 0.1, 0.2, and 0.3 % bile salt. A filter-sterilized cholesterol solution (10 mg/mL in ethanol) was added to the broth to a final concentration of 100 µg/mL, inoculated with 6.0 $\log_{10}$ CFU/mL of strain, and incubated at 37 °C for 18 h. After incubation, the cells were removed by centrifugation for 10 min at 6,000 × *g* at 4 °C. The resulting supernatant was analyzed for remaining cholesterol by a one-step cholesterol test kit (Span Diagnostics, India). The % cholesterol reduction was calculated as $A_{(Initial)} - A_{(Final)} / A_{(Initial)} \times 100$. Growth of bacteria was expressed in $\log_{10}$ CFU/mL and the % survival of strain was then calculated.

Effect of cholesterol on *L. helveticus*

Cell morphology

It was studied by inoculating 1 mL of 18 h old (6.5 $\log_{10}$ CFU/mL) culture in 100 mL MRS medium supplemented with 3 mM cholesterol. Samples were withdrawn at 0, 18, 24, 42, and 48 h incubation at 37 °C and subjected (without staining) for microscopic analysis at 45 x magnification.

Adhesion properties

Bacterial adhesion to solvent

It was measured according to **Rosenberg *et al.* (1980)** with some modifications. The strain was grown at 37 °C for 30 h in MRS medium with and without 3 mM cholesterol. The cells were pelleted at 8,000 × *g* for 5 min and washed twice with PBS, resuspended in 0.1 M KNO_3 (pH 6.2) and A_{600} was measured and recorded as A_0. The 1 mL solvent like chloroform, ethyl acetate, and xylene was added separately to 3 mL of cell suspension. After 10 min pre-incubation at room temperature (28 °C), two phases were mixed by gentle vortexing for 2 min. The aqueous phase was removed after 20 min of incubation at room temperature and A_{600} was measured as A_1. The % bacterial adhesion to solvent was calculated as $(A_0 - A_1/A_0) \times 100$.

Autoaggregation assay

It was performed according to **Del Re *et al.* (2000)** with certain modifications. The strain was grown at 37 °C for 30 h in MRS medium with and without 3 mM cholesterol. The cells were pelleted and washed twice with PBS and resuspended in same to get A_{600} to 0.5 of which 4 mL was mixed by gentle vortexing for 10 s and incubated at 37 °C for 1 h. After incubation, A_{600} of upper suspension was measured. The % autoaggregation was expressed as: $A_0 - (A_t / A_0) \times 100$, where A_t represents the absorbance at time t = 1 h and A_0 the absorbance at t = 0.

RESULTS AND DISCUSSION

Cholesterol biotransformation

Biotransformation was carried out by growing *L. helveticus* at 37 °C up to 120 h in minimal medium supplemented with 3 mM cholesterol as sole carbon source. TLC of 24, 48, and 72 h supernatant showed two spots of which one match with cholesterol and other was unknown (Figure 1). The cholesterol oxidase-like activity was determined up to 120 h to analyze transformation. Intracellular and extracellular cholesterol oxidase-like activity was found to be highest (12 U/mg) at 48 h (Figure 2). The sample showing highest enzyme activity was extracted with ethyl acetate and subjected to GC-MS. Two products of transformation were identified on GC-MS *viz.* cholesta-4, 6-dien-3-ol and cholest-5-en-3-ol (3.beta) with molecular weight of 384.34 and 386.35 (Figure 3). The cholesta-4, 6-dien-3-ol was reported as the oxidized product of cholesterol, since it was formed by radical induced oxidation (**Liu and Shan, 2006**). A spot appeared on TLC at top position was thought to be a mixture of cholesterol and cholest-5-en-3-ol (3.beta), as both showed little structural difference. Both of the transformed products may serve as starting materials for synthesis of metabolites used in production of anabolic drugs and contraceptive hormones. The quantity of transformed products was not determined in this study.

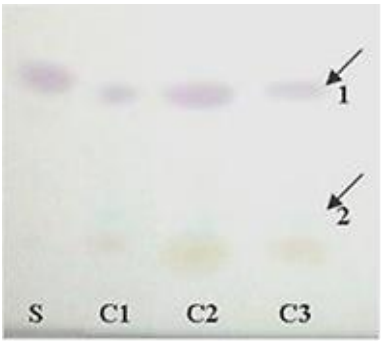

Figure 1 TLC analysis of cholesterol biotransformation by *L. helveticus*. S: standard cholesterol; C1, C2, and C3 are samples taken at 24, 48, and 72 h, arrows indicated 1: cholesterol or mixture of cholesterol and transformed product, 2: transformed product.

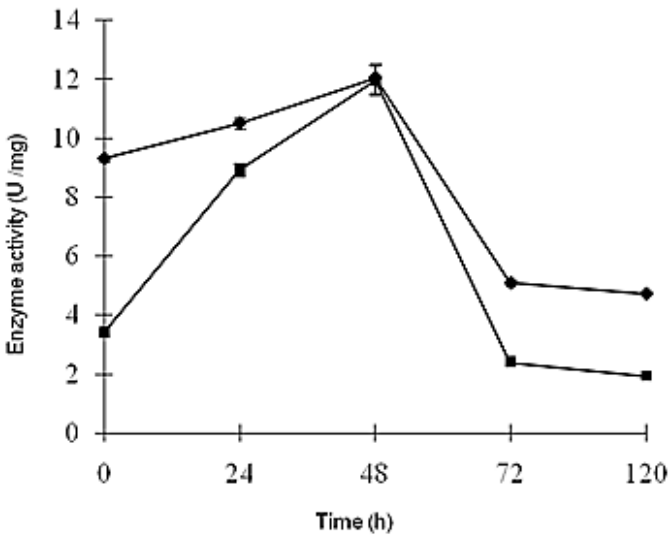

Figure 2 Intracellular (*filled square*) and extracellular (*filled diamond*) cholesterol oxidase-like activity of *L. helveticus* at 37°C for 0 – 120 h incubation in minimal medium containing 3 mM cholesterol. The values represent the means of (n=3) three independent experiments (mean ± standard deviation).

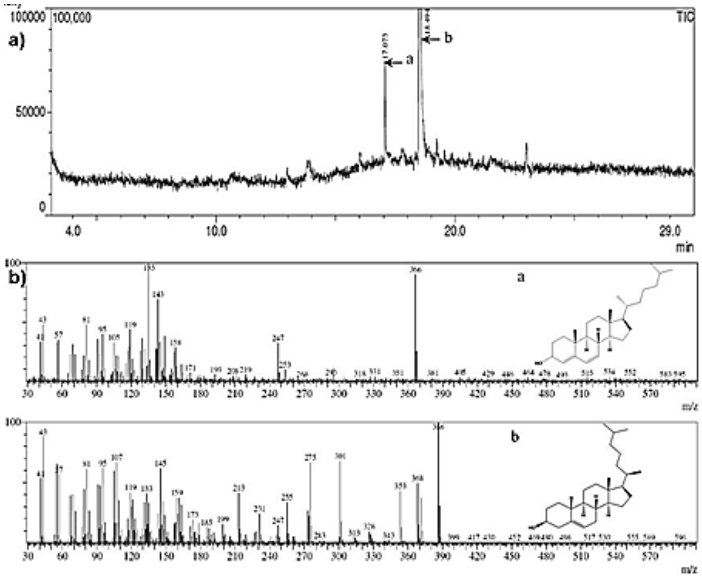

Figure 3 GC-MS analysis of cholesterol transformed products by *L. helveticus*. **a)** Gas chromatogram, arrows indicated retention time of compound a [cholesta-4, 6-dien-3-ol]; b [cholest-5-en-3-ol (3.beta)], **b)** Mass analysis of a [cholesta-4, 6-dien-3-ol]; b [cholest-5-en-3-ol (3.beta)].

In our previous report (**Ahire *et al.*, 2012**), we demonstrated *Lactobacillus helveticus* mediated cholesterol biotransformation to androst-4-ene-3, 17 dione (AD) and androsta-1, 4-diene-3, 17-dione (ADD) in presence of nucleus cleavage inhibitor 2,2′-dipyridyl. However, in this study, we report capability of this strain to transform cholesterol to cholesta-4, 6-dien-3-ol mediated through cholesterol oxidase-like activity in absence of 2,2′-dipyridyl.

Cholesterol assimilation in presence of bile

The strain showed its ability to remove cholesterol from MRS broth with and without bile salt. The maximum cholesterol removal was observed in the medium without bile salt. The medium supplemented with increasing concentration of bile salt found to be inhibitory for cholesterol assimilation (Figure 4). In contrast, bile salt is known for its emulsifying capability for lipid molecules (**Lim *et al.*, 2004**). However, in this study it was observed that when the bile salt concentration increased from 0.1 to 0.3 %, there was a decrease in cholesterol removal since it affects the survival rate adversely.

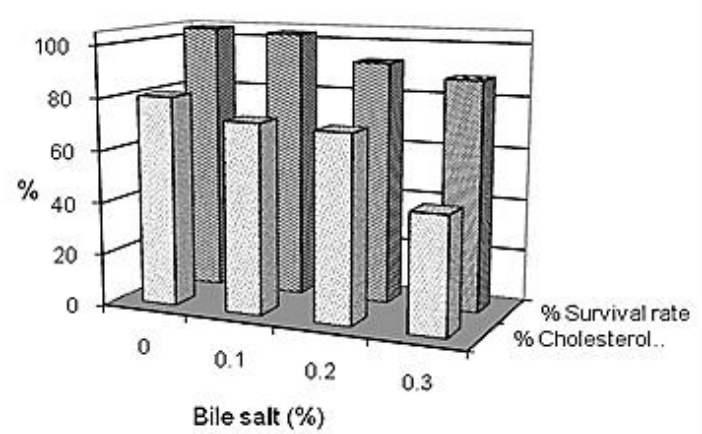

Figure 4 Effect of bile salt on cholesterol assimilation by *L. helveticus* at 37°C for 18 h incubation in MRS medium supplemented with 100 µg /mL cholesterol and bile salt (0.0, 0.1, 0.2, and 0.3%); (n=3).

Effect of cholesterol on *L. helveticus*

Cell morphology

Morphology of cells was observed to be changed as compared to cells grown in MRS medium without cholesterol. The indistinguishable edges of cell wall were observed, while some cells were long and bulgy under the compound microscope (Figure 5). These results clearly showed the modification in cell morphology due to assimilation of cholesterol, while in earlier studies, increase in the number of bacterial cells was observed with increasing incubation time in MRS medium supplemented with 3 mM cholesterol (**Ahire *et al.*, 2012**).

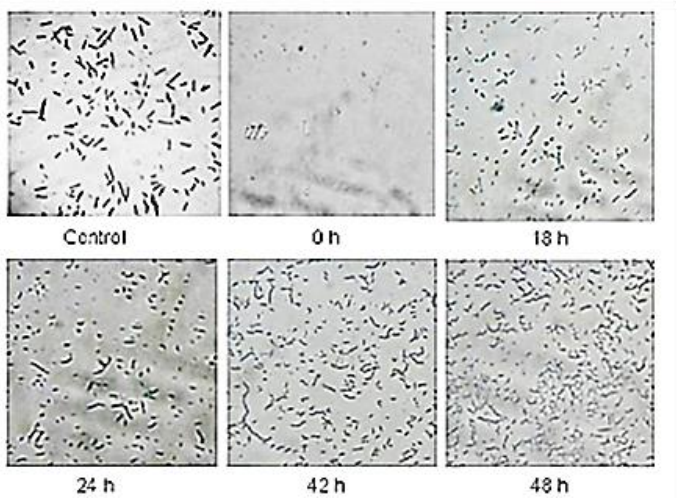

Figure 5 Effect of cholesterol assimilation on cell morphology (45 x magnifications) of *L. helveticus* at 37°C for 0 – 48 h incubation in MRS medium with 3 mM cholesterol, cells of MRS medium used as control.

Adhesion properties

The ability to adhere epithelial cells and mucosal surfaces has been suggested to be an important property of many bacterial strains used as probiotics. The adhesion is initially based on non-specific physical interactions between two surfaces, which then enable specific interactions between proteins and complementary receptors (**Perez *et al.*, 1998**). The study of cell surface hydrophobicity and autoaggregation are the measures of effective colonization in the gastrointestinal tract (**Del Re *et al.*, 2000**). Hence, influence of cholesterol on bacterial adhesion ability was evaluated.

Bacterial adhesion to solvent

It was extensively used for measuring cell surface hydrophobicity in lactic acid bacteria (**Ahire *et al.*, 2013; Collado *et al.*, 2008**). The results of this study demonstrated a great heterogeneity in adhesion to solvents, when strain grown in cholesterol containing MRS medium (Tab 1). The bacterial adhesion to xylene in presence of strong electrolyte 0.1 M KNO_3 (pH 6.2) reflects cell surface hydrophobicity because of absence of electrostatic interactions. The 9 % decrease in hydrophobicity observed in cells grown in cholesterol containing medium, indicating reduction of the adhesion ability of cells due to cholesterol assimilation. The values obtained with the two other solvents, chloroform and ethyl acetate, are regarded as a measure of electron donor/basic and electron acceptor/acidic characteristics of bacterial surface, respectively (**Bellon-Fontaine *et al.*, 1996**). The significant difference in % adhesion of strain to ethyl acetate was observed when a strain grown in cholesterol containing medium. These results are indicative of a decrease in electron acceptor properties of bacterial cell surface.

Table 1 The % microbial adhesion to solvent by *L. helveticus*

Solvent	% Hydrophobicity	
	MRS broth	MRS + 3 mM cholesterol
Xylene	68.49 ± 0.06	59.45 ± 0.32
Chloroform	64.65 ± 0.06	56.99 ± 0.34
Ethyl acetate	76.87 ± 0.06	07.49 ± 0.74

The values represent the means of (n=3) three independent experiments (mean ± standard deviation).

Autoaggregation assay

Autoaggregation ability is one of the important measures to predict adhesion capabilities of probiotic bacteria. The autoaggregation and hydrophobicity of strains are mainly associated with proteinaceous components of cell surfaces (**Nikolic *et al.*, 2010; Kos *et al.*, 2003**). In the present study, lower autoaggregation values were recorded for cells grown in the presence of cholesterol (20.11 ± 0.20 %) as compared to the cells grown in the absence of cholesterol (33.54 ± 1.02 %). The results are in accordance with the decrease in hydrophobicity of strain to xylene in previous experiment. However, the mechanism behind the decrease in autoaggregation and cell surface hydrophobicity in response to cholesterol assimilation is not well understood. These studies suggested that the assimilation of cholesterol by cells might be

decreasing adhesin protein content by modifying cell membrane or masking the responsible receptors.

CONCLUSION

Based on the findings of the present study, strain *Lactobacillus helveticus* showed cholesterol oxidase-like enzyme mediated cholesterol biotransformation in to cholesta-4, 6-dien-3-ol. The cholesterol assimilation in the presence of bile salt and decrease in hydrophobicity on assimilation demonstrated possible cholesterol lowering through elimination of cells in stool which may serve as a promising solution to avoid subsequent health hazards, however further research is required to validate it *in vivo*.

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