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In vitro Antioxidant Potential of Cell Free Supernatant of Probiotic Bacteria

Vandana Bharti [1]*, Archana Mehta [1], Gajendra Kumar Mourya [2] and Laxmi Ahirwal [1]

[1] Lab of Molecular Biology, Department of Botany,
Dr. Hari Singh Gour University (Central University), Sagar, MP, India
[2] Department of Biomedical Engineering, School of Technology,
North Eastern Hill University, Shillong, Meghalaya, India

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Abstract: Probiotic micro-organisms such as lactic acid bacteria are very useful antioxidants which have potential to reduce free radicals in human body. The aim of the present study was to determine the *in vitro* antioxidant potential of cell free supernatant (CFS) of two probiotic bacteria i.e. *Lactobacillus plantarum* KP894100 and *Lactobacillus acidophilus* KP942831. The CFS of *L. plantarum* KP894100 and *L. acidophilus* KP942831 showed potent DPPH, superoxide and hydroxyl radical scavenging activity with concentration dependent manner when compared with ascorbic acids as standard drug. Protein contents present in CFS of *L. plantarum* KP894100 was 12 mg/ml and in *L. acidophilus* KP942831 was 8 mg/ml was determined by Lowry methods.

Key words: *Lactobacillus plantarum*, *Lactobacillus acidophilus, in-vitro,* antioxidant activity, probiotics.

Introduction

Oxidation is a needed reaction in an organism's metabolism. When antioxidants present in human body are unable to hold high amount of free radicals this state is known as oxidative stress. These radicals are unstable molecules that destroy cellular membranes; enzymes and DNA. They are responsible for causing certain disease such as cancer and heart disease [16]. Several microbes such as lactic acid bacteria have potential to reduce free radicals in human body. Lactic Acid Bacteria (LAB) are mostly used as probiotic microorganisms such as *Lactobacillus* sp., *Bifidobacterium* sp., and *Enterococcus* sp. *Lactobacillus* etc. are capable of passing through upper gastrointestinal tract and colonizing in the large intestine. They are the most important group of bacteria inhabiting a wide variety of environmental niches, habitants of mucosal surfaces, particularly the gastrointestinal tract, also present in vegetables, plants, food materials such as grains, cereals, pickles, milk and meat products [18]. They can survive passage through stomach in an active form. LAB are considered industrially important organisms because of their fermentative ability as well as health and nutritional benefits. Moreover, they are generally regarded as safe (GRAS) for incorporation into food products [23]. The objective of this study was to investigate the *in vitro* antioxidant activity of *Lactobacillus plantarum* KP894100 and *L. acidophilus* KP942831 probiotic bacteria.

Materials and methods

Chemicals and reagents

2,2-diphenylpicrylhydrazyl (5 mg), Ethanol,

*Corresponding author (Vandana Bharti)
E-mail: < vandanabharti55@gmail.com >

Ascorbic acid, Pyrogallol (25 mmol/L), Tris-HCl-EDTA buffer (0.05 M, pH 8.20), Ascorbic acid, Distilled water, 1, 10- Phenanthroline, Sodium phosphate buffer, Ferrous sulphate (0.75 mmol/l), Ascorbic acid, acetonitrile and formic acid.

Bacterial culture

Two probiotic bacterial culture of *Lactobacillus plantarum* KP894100 and *Lactobacillus acidophilus* KP942831 were isolated from dairy products. In our previous paper we reported that the, both bacteria showed antimicrobials, antifungal, antihelminthic, anticancer and antidiabetic activity [24-27]. Hence, in this study, we selected the above mentioned microbial strains for *in vitro* antioxidant potential.

Production of cell free supernatant

To obtain Cell Free Supernatant (CFS), one loop full of bacterial culture transferred in to 10 ml MRS broth medium under aseptic condition and incubated at 37°C for 24 h in bacteriological incubator (REMI). 10 ml of this overnight grown culture of bacteria (known as inoculum) were used to inoculate 400 ml of MRS broth and incubated under micro aerobic conditions at 37°C for 72 h. Aliquots were withdrawn at intervals of 6 h and examined for growth (OD 600 nm) and pH. 10 ml aliquot was centrifuged at 10,000 rpm for 10 min and the supernatant passed through the sterile membrane filter (0.45 µm pore size). 50 µl of CFS was used to detect the *in vitro* antioxidant activity [29].

***In vitro* antioxidant activity**

Free radical scavenging activity by DPPH method

The reaction mixture contained 1 ml of each concentrations of 10, 50, 100, 250, 500 (µl/ml) of CFS and mixed with 2 ml of ethanolic solution of DPPH (0.1 mM). The reaction mixtures were allowed to stand at room temperature for 20 min. The absorbance of the samples was measured at 517 nm. Reagent solution without test samples was used as control. Ascorbic acids were used as standard drug [13]. The DPPH free radical scavenging activity was calculated using the following formula:

% Radical scavenging activity = Control OD - Sample OD / Control OD X 100

Scavenging activity of superoxide anion radical

The superoxide radical scavenging activity was estimated by pyrogallol autoxidation according to the method given by Wang *et al*., [28] with some modifications. The reaction mixture contained 3 ml of Tris-HCl-EDTA buffer (0.05 M, pH 8.2) and 1ml of CFS sample each concentrations (10, 50, 100, 250, 500 µl/ml) were mixed and incubated for 25 min in a water bath at 25°C. Instantly after that, 40 µl of Pyrogallol (preheated at 25°C) solution (25 mmol/l) was added. The optical density (OD) was measured at 325 nm for every 5 min till 30 min against a blank by using UV-VIS spectrophotometer. Distilled water used as blank whereas ascorbic acids used as control. Scavenging activity was measured using the following equation:

% Superoxideradical scavenging activity = Sample OD - Control OD / Control OD X 100

Hydroxyl radical scavenging activity

The assay of hydroxyl radical scavenging activity was analyzed by the method of Kanmani *et al*. [9]. The reaction mixture contained 1 ml of CFS of different concentration (10, 50, 100, 250, 500 µl/ml), 1 ml of 1,10-phenanthroline (0.75 mM), 1.5 ml of sodium phosphate buffer (0.15M, pH 7.4), 1 ml of $FeSO_4$ (0.75 mM) and 1 ml of H_2O_2 (0.01 %, v/v). The mixture was incubated at 37°C for 30 min then the absorbance of the mixture was measured at 536 nm. Ascorbic acid was used as the control. The scavenging activity on hydroxyl radical was calculated using the following formula:

% Hydroxyl radical scavenging activity = Sample OD - Control OD / Control OD X 10

Protein estimation

Concentration of protein in cell free supernatant (CFS) was determined by Lowry's method, where in 0.5 ml of enzyme solution was made upto 1ml using distilled water and reacted with Lowry's reagent as per the protocol [15].

Results

DPPH scavenging activity

The DPPH radical scavenging activity of cell free supernatant of bacterial strain *L. plantarum* and *L. acidophilus* showed potent activity with concentration dependent manner when compared with ascorbic acids standard drug. The CFS of *L. plantarum* showed 4.33 ± 0.5, 16.60 ± 1.2, 34.43 ± 2.4, 68.60 ± 5.6 and 93.40 ± 7.4 percentage inhibition of DPPH free radical scavenging activity while CFS of *L. acidophilus* showed 3.37 ± 0.4, 14.30 ± 1.1, 33.33 ± 2.4, 64.33 ± 5.6 and 83.13 ± 6.6 percentage inhibition of DPPH free radical scavenging activity at the concentration of 10, 50, 100, 250, and 500 µl/ml respectively (Table 1).

Superoxide radical scavenging activity

The cell free supernatant of bacterial strain *L. plantarum* and *L. acidophilus* showed potent inhibitory superoxide radical scavenging activity with increasing concentration compared with ascorbic as acids. The inhibition percent of superoxide radical scavenging of CFS of *L. plantarum* were 0.040 ± 0.007, 0.2160 ± 024, 0.385 ± 0.037, 0.532 ± 0.067 and 0.765 ± 0.085 while CFS of *L. acidophilus* showed 0.022 ± 0.004, 0.112 ± 0.013, 0.245 ± 0.027, 0.424 ± 0.042 and 0.616 ± 0.07 percent inhibition of superoxide radical scavenging activity at the concentration of 10, 50, 100, 250 and 500 µl/ml respectively (Table 2).

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of cell free supernatant of bacterial strain *L. plantarum* and *L. acidophilus* showed potent activity with increasing concentration when compared with ascorbic acids. The CFS of *L. plantarum* showed 19.6 ± 1.8, 28 ± 2.7, 44.04 ± 3.2, 59.76 ± 4.6 and 73.59 ± 5.9 percent inhibition of hydroxyl radical scavenging activity while CFS of *L. acidophilus* showed 27 ± 2.8, 36 ± 2.5, 56 ± 4.4, 75.46 ± 6.1 and 84.56 ± 6.5 percentage inhibition of hydroxyl radical scavenging activity at the concentration of 10, 50, 100, 250 and 500 µl/ml respectively (Table 3).

Table 1. Result of DPPH scavenging activity of *L. plantarum* and *L. acidophilus*.

	Strain		
Concentration (µl/ml)	***L. plantarum***	***L. acidophilus***	**Ascorbic acid**
10	4.33 ± 0.5	3.37 ± 0.4	27.96 ± 1.4
50	16.60 ± 1.2	14.30 ± 1.1	52.00 ± 4.3
100	34.43 ± 2.4	33.33 ± 2.4	78.00 ± 6.0
250	68.60 ± 5.6	64.33 ± 5.6	84.36 ± 6.5
500	93.40 ± 7.4	83.13 ± 6.6	96.90 ± 8.0

The results were expressed in % inhibition as Mean ± S.D. of triplicates

Table 2. Result of superoxide radical scavenging activity *L. plantarum* and *L. acidophilus*

	Strain		
Concentration (µl/ml)	***L. plantarum***	***L. acidophilus***	**Ascorbic acid**
10	0.040 ± 0.007	0.022 ± 0.004	0.014 ± 0.002
50	0.2160 ±. 024	0.112 ± 0.013	0.218 ± 0.022
100	0.385 ± 0.037	0.245 ± 0.027	0.432 ± 0.043
250	0.532 ± 0.067	0.424 ± 0.042	0.748 ± 0.084
500	0.765 ± 0.085	0.616 ± 0.072	1.476 ± 0.120

The results were expressed in % inhibition as Mean ± S.D. of triplicates

Table 3. Result of hydroxyl radical scavenging activity of *L. plantarum* and *L. acidophilus*

Concentration (µl/ml)	Isolates		
	L. plantarum	***L. acidophilus***	**Ascorbic acid**
10	19.6 ± 1.8	27 ± 2.8	27.74 ± 1.3
50	28 ± 2.7	36 ± 2.5	52.03 ± 2.5
100	44.04 ± 3.2	56 ± 4.4	73.39 ± 4.1
250	59.76 ± 4.6	75.46 ± 6.1	86.72 ± 4.9
500	73.59 ± 5.9	84.56 ± 6.5	94.54 ± 4.3

The results were expressed in % inhibition as Mean ± S.D. of triplicates

Table 4. Quantitative estimation of protein in CFS of *L. plantarum* and *L. acidophilus*

Strain	Volume (ml)	Amount of protein (mg/ml)	
		CFS	**Standard (BSA)**
L. plantarum	100	12	30
L. acidophilus	100	8	30

CFS: cell free supernatant, BSA: bovine serum albumin

Protein estimation

Quantitative protein estimation in CFS was analyzed by Lowry method. Protein content in CFS of *L. plantarum* was 12 mg/ml and in *L. acidophilus* was 8 mg/ml respectively (Table 4). The CFS of *L. plantarum* was found to have highest protein concentration (12 mg/ml) and lowest was in CFS of *L. acidophilus* (8 mg/ml).

The applications of LAB have already been proved safety profile and efficiency of promising future of GRAS organisms as the expression system of choice that can revolutionize the field of recombinant protein production [6]. The inhibitory substances produced by bacteria can be generally proteins [22]. The proteinaceous nature of the *L. plantarum* and *L. acidophillus* for antimicrobial activity was clearly understood by the quantitative analysis of protein present in it.

Hemalatha and Shanthi [7] stated that the antimicrobial activity of *B. subtilis* is due to their proteinaceous nature. Barefoot *et al.*,[4] purified cell-free proteins with a pI of 4.1 and a molecular size of 58 kDa by chromatofocusing and gel filtration high-performance liquid chromatography method that induce lactacin B production in lactic acid bacteria.

Discussion

Oxidation is a needed reaction in an organism's metabolism. When high amount of free radicals present in human body these states is known as Oxidative stress. These radicals are unstable molecules that destroy cellular membranes; enzymes and DNA. They are responsible for causing certain disease such as cancer and heart disease [16]. Microbes like lactic acid bacteria have potential to reduce free radicals in human body.

Antioxidative ability of different strain of *L. acidophilus*, *L. bulgaricus*, *S. thermophilus* and *B. longum* showed the highest reducing activity [12].

DPPH radical reacts with an antioxidant compound that can donate hydrogen and gets reduced; it is converted into diphenylpicryl hydrazine. This can be identified by the conversion of purple to light yellow colour. Antioxidants react with DPPH which is a stable free radical and is reduced to the DPPH-H and as a consequence the absorbance is decreased. The degree of coloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. DPPH radicals react with suitable reducing agents as a result the electrons become paired and form the corresponding hydrazine [3]. The

exopolysaccharides of *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus plantarum* serve as primary antioxidant [14]. Strain *Bacillus amyloliquefaciens* exhibited a significant dose dependent inhibition of DPPH activity with the highest radical-scavenging activity (67.33 %) at 10^8 cfu/ml [8].

Among reactive oxygen species, hydroxyl radical is the most reactive and it can react with all bio macromolecules in living cells resulting in severe damage to the adjacent macro molecules. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell. The neutral exopolysaccharide (LPC-1), isolated from the culture of *Lactobacillus plantarum* C88 had good scavenging ability on hydroxyl radicals [30]. Venkatesan *et al*., [19] stated that the different concentrations of probiotic species of *Bifidobacterium* sp., *Lactobacillus* sp. and *S. cerevisiae* showed strongest radical scavenging assay.

The DPPH and ABTS radicals scavenging activity of *Bifidobacterium* and *Propionebacterium* showed the maximum DPPH scavenging potential of cell free extract with *Propionebacterium freudenreichii* (97.75 %) and it was significant increase when compared with vitamin C and BHT followed by *L. reuteri* (96.74 %) activity [1]. Nedelcheva *et al*.,[17] investigated antioxidant activity of *L. plantarum* NBIMCC 2415, which showed high antioxidant activity at 10^9cfu/g against peroxide radicals. Sah *et al*.,[20] showed antioxidant and strong antimutagenic activities of crude peptide extract isolated from *L. acidophilus*, *L. casei* and *L. paracasei subsp. Paracasei*. These peptides contain high radical scavenging activities with 1,1-diphenyl-2- picrylhydrazyl and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) respectively and strong antimutagenicity (26.35 %) *in vivo*. According to Klayraung and Okonogi, [10] the supernatant of strains *L. fermentum* FTL2311 and *L. fermentum* FTL10BR showed high free radical scavenging activity.

L. fermentum strains suggested a high efficacy of these strains as promising probiotics with potential antioxidant activity for health promotion of the host. Songisepp *et al*., [21] evaluated the functional efficacy of the probiotic *L. fermentum* ME-3 the oxidative stress significantly improved blood TAA (Total Antioxidative Activity) and TAS (Total Antioxidative Status) that was proved by reduction of the oxidative stress of blood and urine of healthy volunteers. Annuk *et al*.,[2] found similar result of goats' milk fermented with *L. fermentum* ME-3.

Conclusion

Probiotic have antioxidant activity which improve the function of biochemical reaction in biological system. It is found to be used as a treatment for minor illnesses and serious life threatening issues as well as a preventative dietary supplement. The cell free supernatant of lactic acid bacteria have a long history and are widely used as a health food supplement and also used in pharmaceutical industries. These two isolated bacteria have antimicrobials, antifungal, antihelminthic, anticancer and antidiabetic activity. Moreover, to use as probiotic the *in vitro* antioxidant activity is done. In the present study two species of lactobacillus i.e. *L. plantarum* KP894100 and *L. acidophilus* KP942831 showed DPPH, Hydroxyl, superoxide scavenging radical activity. On the basis of our findings it can be concluded that these bacteria have the potential to use as probiotic bacteria and can be further analyzed for medical applications.

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