LWT - Food Science and Technology 61 (2015) 263–268

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Research note

Antioxidant activity and γ-aminobutyric acid (GABA) producing ability of probiotic *Lactobacillus plantarum* DM5 isolated from Marcha of Sikkim

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ARTICLE INFO

Article history:
Received 21 August 2013
Received in revised form
25 August 2014
Accepted 5 November 2014
Available online 13 November 2014

Keywords:
Lactobacillus plantarum
Antioxidant activity
DPPH
γ-Aminobutyric acid
Probiotic

ABSTRACT

A probiotic *Lactobacillus plantarum* DM5 isolated from fermented beverage Marcha of Sikkim displayed antioxidant properties. The antioxidant properties of *L. plantarum* DM5 were compared with *L. plantarum* NRRL B-4496 and *Lactobacillus acidophilus* NRRL B-4495. A concentration of 10^{10} CFU/ml of *L. plantarum* DM5 demonstrated the hydroxyl radical, superoxide anion radical and DPPH scavenging activities of 49%, 48%, and 55%, respectively and reducing activity of 149 μM (cysteine equivalents). Comparative analysis *L. plantarum* DM5 exhibited 38% and 20% higher hydroxyl radical, 31% and 22% higher superoxide anion radical, 43% and 33% higher DPPH scavenging activities than *L. plantarum* and *L. acidophilus*, respectively. Probiotic *L. plantarum* DM5 has the ability to produce bioactive γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in mammalian brain. It also possessed the ability to multiply in MRS medium containing abundant hydrogen peroxide (1 mM) and exhibited 20% inhibition rates of ascorbate autoxidation at 10^{10} CFU/ml. These findings suggested that *L. plantarum* DM5 has the potential to protect the oxidative damage mediated by the reactive oxygen species and can act as an antioxidative probiotic.

1. Introduction

Environmental pollution, UV radiation and several normal metabolic processes have been associated with the induction of high level of reactive oxygen species (ROS) such as superoxide anion and hydroxyl radical in mammalian cells. Elevated levels of these highly active free radicals have been found to be associated with numerous human diseases, such as carcinogenesis, atherosclerosis, Alzheimer's disease, ageing and degenerative processes (Hazra, Biswas, & Mandal, 2008; Lin & Yen, 1999). Although mammalian cells acquire efficient antioxidant defence and repair systems which have evolved to protect them against oxidative damage but, these systems are not effective enough to completely prevent the damage (Lin & Yen, 1999; Simic, 1988). Antioxidants are oxidizing agents which protect the oxidation of cellular oxidizable substrates by scavenging free radicals and ROS (Lee et al., 2010). Various synthetic and natural antioxidants have been reported such as, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ), however, there are several doubts about the safety and long-term effects of synthetic antioxidants on health (Kumar, Ganesan, & Rao, 2008). Therefore, antioxidants from natural sources are more desirable. It has been shown that some *Lactobacilli* possess antioxidative activity and are able to decrease the risk of accumulation of ROS (Kaizu, Sasaki, Nakajima, & Suzuki, 1993) and degrade the superoxide anion and hydrogen peroxide (Kullisaar et al., 2002). A fermented sea tangle solution with *Lactobacillus brevis* BJ20 exhibited strong DPPH scavenging and superoxide radical scavenging activities (Lee et al., 2010). *Lactobacillus plantarum* 7FM10 isolated from the traditional Japanese food narezushi (Kanno, Kuda, An, Takahashi, & Kimura, 2012) and *L. plantarum* isolated from Chinese fermented food (Li et al., 2012) displayed DPPH, hydroxyl and superoxide radical scavenging capacities.

γ-Aminobutyric acid (GABA) is an ubiquitous non protein amino acid produced by α-decarboxylation of glutamate by glutamate decarboxylase and acts as a major inhibitory neurotransmitter in the mammalian central nervous system (Schousboe & Waagepetersen, 2007). In addition, GABA has hypotensive, tranquilizing and diuretic effects and can prevent diabetes (Hayakawa et al., 2004; Li & Cao, 2010). The enzyme glutamate decarboxylase is largely distributed in higher plants, animals and bacteria.

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http://dx.doi.org/10.1016/j.lwt.2014.11.013
0023-6438/

Recently some of Lactobacillus spp. such as *L. brevis*, *L. plantarum*, *Lactobacillus delbrueckii*, *Lactobacillus paracasei* and *Lactococcus lactis* have shown glutamate decarboxylase activity and ability to produce GABA in the concentration range 10–350 mmol/L depending upon the concentration of monosodium glutamate (MSG) in the fermentation medium (Di Cagno et al., 2010; Li & Cao, 2010). The traditional fermented food sources enriched in glutamate are important sources for isolation of GABA-producing lactic acid bacteria (Di Cagno et al., 2010; Lee et al., 2010). So far, not much information is available on the antioxidant activities and GABA producing ability of *Lactobacillus* strains isolated from ethnic fermented food products of India. Therefore, in the present study bacteriocin producing probiotic *L. plantarum* isolated from traditional fermented beverage Marcha of Sikkim (Das & Goyal, 2014) was examined for its *in vitro* antioxidant activities including inhibition of ascorbate autooxidation, the scavenging of DPPH radicals, superoxide anion radicals and hydroxyl radicals. The GABA producing ability of *L. plantarum* DM5 was also explored for further establishing it as an exclusive probiotic anti-oxidative strain.

2. Materials and methods

2.1. Bacterial strains and culturing

L. plantarum DM5 isolated from traditional fermented beverage Marcha of Sikkim was selected on the basis of *in vitro* probiotic properties (Das & Goyal, 2014). The strains *L. acidophilus* NRRL B-4495 and *L. plantarum* NRRL B-4496 were procured from ARS Culture Collection, USA and maintained in MRS medium (De Man, Ragosa, & Sharpe, 1960).

2.2. Resistance of Lactobacillus strains to hydrogen peroxide

The hydrogen peroxide resistance ability of *Lactobacillus* strains was assessed by the method of Li et al., 2012 with some modifications. The overnight grown cultures of *Lactobacillus* strains were inoculated at 1% (v/v) into 100 ml of MRS medium (control) and 100 ml MRS medium containing 0.4, 0.8 or 1.0 mmol/L hydrogen peroxide (50%, Merck, India) and incubated at 37 °C for 8 h. The cell growth was measured by taking absorbance at a 600 nm (A_{600}) by a UV spectrophotometer (Cary 100, Varian, USA).

2.3. In vitro assessment of antioxidant activity of Lactobacillus strains

2.3.1. Preparation of cells and cell free extracts

The *Lactobacillus* strains were harvested after 20 h incubation by centrifugation (8,000*g*, 4 °C for 10 min). The bacterial cells were washed twice with deionised water and re-suspended in deionised water to make the bacterial cell counts to 10^6, 10^8 and 10^{10} CFU/ml. The cell-free extracts were prepared by incubating the cells (10^6, 10^8 or 10^{10} CFU/ml) at 37 °C for 30 min in presence of 1 mg/ml lysozyme following the method of Li et al. (2012). The sonication was performed by a sonicator (Vibra cell, SONICS, Newtown, CT, USA) for 10 min (1 min on/1 min off pulse; at 35% amplitude) and the cell debris was removed by centrifugation (10,000*g*, 4 °C for 150 min).

2.3.2. Assay of scavenging activity against DPPH radical

The DPPH (1-diphenyl-2-picrylhydrazyl) scavenging activity was measured by the method of Lee et al. (2010). The 100 μl of ethanolic DPPH solution (0.4 mmol/L) was mixed vigorously with 100 μl of intact cells (10^6, 10^8 and 10^{10} CFU/ml) or water (control) and incubated at 37 °C in the dark for 30 min. The absorbance of the mixture was measured at 517 nm using a microplate reader (Infinite 200 Pro, Tecan, Switzerland). The scavenging activity was calculated as; Scavenging activity (%) = $[1\text{-}(A_s - A_b)/A_c] \times 100$, where A_b, A_c and A_s is the absorbance of the blank (ethanol and the cells), control (deionised water) and the sample, respectively.

2.3.3. Assay of inhibition of ascorbate autoxidation

The antioxidative activity of *Lactobacillus* strains was also assayed by the method of inhibition of ascorbate autoxidation (Lin & Yen, 1999). The cell free extract of 0.1 ml of different cell concentrations (10^6, 10^8 or 10^{10} CFU/ml) or distilled water (as control) was mixed with 9.8 ml of 0.2 mol/L sodium phosphate buffer (pH 7.0) and 0.1 ml of 5 mmol/L ascorbate solution (Sigma Aldrich, St. Louis, USA). The 1 ml reaction mixture was then transferred to a cuvette and the absorbance at 265 nm was measured for 10 min at 37 °C. Inhibition of ascorbate autoxidation was calculated according to the following equation; Inhibition rate (%) = $[(A_c - A_s/A_c)] \times 100$, where A_c and A_s is the absorbance of the control and sample, respectively.

2.3.4. Assay of reducing activity

Reducing activity of the *Lactobacillus* stains was determined according to the method of Lin & Yen (1999). A 0.2 ml sample or distilled water (as control) was mixed with 0.2 ml of 1% potassium ferricyanide and 0.2 ml of 20 mmol/L sodium phosphate buffer (pH 7.0) and was incubated at 50 °C for 20 min. The reaction was stopped by adding 0.2 ml of 10% (w/v) trichloroacetic acid and was centrifuged at 780 *g* at 4 °C for 10 min. The upper layer of supernatant (0.5 ml) was mixed with 0.1 ml of 0.1% (w/v) ferrichloride and 0.4 ml of distilled water. The absorbance at 700 nm (A_{700}) was measured and L-cysteine was used as a standard.

2.3.5. Assay of scavenging activity against superoxide anion radical

Superoxide anion radical scavenging activity was estimated by the reduction of nitrobluetetrazolium (NBT) according to a method previously reported by Wang, Yu, & Chou (2006). The 1.0 ml reaction mixture containing 20 mmol/L sodium phosphate buffer (pH 7.4), 50 μmol/L NBT (Sigma Aldrich, St. Louis, USA), 75 μmol/L nicotinamide adenine dinucleotide (NADH), 15 μmol/L phenazine methosulfate (PMS) (Sigma Aldrich, St. Louis, USA) and 50 μl of sample (cell free extract) or distilled water (control) was incubated at 37 °C for 5 min and the absorbance at 560 nm was measured. Superoxide anion radical scavenging activity was calculated as; Scavenging activity (%) = $[(A_c - A_s/A_c)] \times 100$, where A_c and A_s is the absorbance of the control and sample, respectively.

2.3.6. Assay of scavenging activity against hydroxyl radical

The hydroxyl radical scavenging assay was conducted using the method of He, Luo, Cao, & Cui, 2004. The 1.0 ml of reaction mixture containing 20 mmol/L sodium phosphate buffer (pH 7.4), 0.435 mmol/L brilliant green, 0.5 mmol/L ferrous sulphate heptahydrate ($FeSO_4 .7H_2O$), 3.0% (w/v) H_2O_2 was mixed with 100 μl of cell-free extract of *Lactobacillus* strains or distilled water (control). The reaction mixture was incubated at 37 °C for 20 min and the absorbance was measured at 624 nm. The scavenging ability was calculated using the following equation; Scavenging activity (%) = $[(A_s - A_c/A_b - A_c)] \times 100$, where A_s is the absorbance in the presence of the sample, A_c represents the absorbance of the control solution and A_b is the absorbance of blank without the sample and Fenton reaction system.

2.4. Identification of GABA producing ability of L. plantarum DM5 by thin layer chromatography

In order to evaluate the GABA-producing ability of *L. plantarum* DM5 and other two standard strains *L. acidophilus* NRRL B-4495