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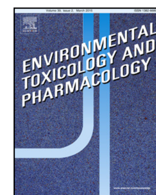

Characterization of a cadmium resistance *Lactococcus lactis* subsp. *lactis* strain by antioxidant assays and proteome profiles methods

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ABSTRACT

Heavy metal contamination poses a major threat to the environment and human health for their potential toxicity and non-biodegradable properties. At present, some probiotics bacteria are reported to have great potential to eliminate heavy metals from food and water. In this study, resistance properties of a newly isolated *Lactococcus lactis* subsp. *lactis* for cadmium were studied by antioxidant assays and proteomics analysis. Antioxidant capacity of this strain was significantly activated under cadmium stress indicated by Fenton reaction, DPPH assay, SOD assay and GSH assay. Intracellular antioxidant enzyme systems, such as superoxide dismutase, glutathione reductase and catalase were suggested to play vital roles in the activated antioxidant capacity. The up-regulated *cad*A was associated with the activated P-type ATPases that plays an important role in cadmium resistance. Proteomics analysis identified 12 over-expressed proteins under 50 mg/L cadmium stress and these proteins are abundant in oxidative stress response and energy metabolism regulation, which were considered as consequences as cadmium resistance of the strain. Thus, the probiotics *Lactococcus lactis* subsp. *lactis* may resist cadmium stress through antioxidant approach and enhanced energy metabolism. The food grade *lactis* strain may be applied in metal decontamination in environment and food/feed.

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1. Introduction

Heavy metal contamination poses a major threat to the environment and human health for their potential toxicity and non-biodegradable properties. Cadmium (Cd) is a hazardous heavy metal and one of the IARC Group I carcinogens (Mcmurray and Tainer, 2003) for causing a grossly biological impact on plants, animals and humans by bio-accumulation and bio-concentration process (Bhakta et al., 2012). Exposure to low level of Cd for human can result in renal injury, liver damage, cardiovascular system diseases and lung cancer (Stayner et al., 1992; Compare et al., 2011). Industrial emissions and phosphate fertilizers are considered to be responsible for the increasing levels of cadmium in the environment, which in turn can lead to an elevated uptake of Cd by the crop (Ernst et al., 1992) and aquatic organism. Various freshwater fishes (Yousafzai et al., 2010) and marine fishes (Velusamy et al., 2014) have been reported to accumulate heavy metals in the body through the consumption of water and food.

Both environmental safety and food/feed safety are threatened by heavy metals contamination. Traditional physical and chemical approaches are not appropriate for removing heavy metals from contaminated food/feed for potential secondary contamination. Some food grade probiotics, such as *lactic acid* bacteria are revealed to be promising in eliminating heavy metals contamination (Marc et al., 2012). A cadmium resistance probiotics, *Lactococcus lactis* subsp. *lactis*, was successfully isolated from pickle that poses significant cadmium resistance properties and commendable prospects for application.

Efforts have been made to understand the mechanisms by which cells response to when exposed to exogenous metals. Antioxidant approach is considered as one of the most important mechanisms by which cells resisting metal ions (Tito et al., 2011). Elevated concentration of metals is known to trigger adverse redox-reactions in the cell, such as generation of toxic hydroxyl radicals, reactive oxygen species (ROS) in the reaction. The radicals formed are highly reactive molecules and play roles in a number of deleterious reactions, such as peroxidation of lipids, which causes membrane

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Table 1
Broad spectrum heavy metal resistance of *Lactococcus lactis* subsp. *lactis*.

Heavy metals	Concentration (mg/L)	OD_{600}	Incubation time (h)
Cr (VI) (K_2CrO_4)	100	1.072	24
Ag^+ ($AgNO_3$)	100	1.044	24
Ni^{2+} ($NiSO_4$)	100	0.008	24
Ni^{2+} ($NiSO_4$)	50	0.348	24
Cr^{3+} ($CrCl_3$)	100	0.904	24
Mg^{2+} ($MgCl_2$)	100	0.816	24
Cu^{2+} ($CuSO_4$)	100	0.272	24
Mn^{2+} ($MnCl_2$)	100	0.88	24
Mn^{2+} ($KMnO_4$)	100	0.852	24
Fe^{3+} ($FeCl_3$)	100	0.916	24
Zn^{2+} ($ZnSO_4$)	100	0.876	24
Ca^{2+} ($CaCl_2$)	100	1.032	24
Mo (VI) (Na_2MoO_4)	100	0.956	24

disruption and the oxidative denature of proteins (Gieseg et al., 2000).

The rapidly evolving of proteomics is directed toward providing a comprehensive view of the characteristics of cellular proteins (Anderson and Anderson, 1998). Rather than being hypothesis-driven where subsequent studies are directed based on previous findings and specific results are anticipated, proteomics is largely discovery-driven where newly acquired data provides details about the system under study and independent of predictable results (Pandey and Mann, 2000; Patterson and Aebersold, 2003). The goals of conducting proteomics are to characterize differentially expressed proteins and further understand how cells function under conditions of metal stress.

In this study, cadmium resistance properties of the newly isolated probiotics, *Lactococcus lactis* subsp. *lactis*, were evaluated by antioxidant capacity and proteome profiles. Fenton reaction, DPPH assay, SOD assay and GSH assay were conducted to study the antioxidant properties of *lactis*. In addition, P-type ATPase assays were performed to study the relationship between energy metabolism and metal stress. Bidimensional electrophoresis (2-DE) and mass spectrometry combined with real time quantitative RT-PCR analysis were conducted to reveal the changes of proteomics of *lactis* under cadmium stress.

2. Materials and methods

2.1. Materials

Lactococcus lactis subsp. *lactis* with cadmium resistance was isolated from pickle with cadmium stress and the detailed method was shown in Supplementary material S1. The control strain, *Lactococcus lactis* ML23 (Zhang et al., 2010), was provided by professor Hongtao Tian, Hebei Agricultural University, China.

2.2. Broad spectrum heavy metal resistance detection

Resistance properties of the strain for cadmium and other heavy metals, such as Pb^{2+}, Cr(VI), Ag^+, Mg^{2+}, Mn^{2+}, Mn^{7+}, Na^+ and Ca^{2+} were evaluated using growth curves under different metals stresses (Table 1). The strain was cultivated at MRS medium (Merck-Darmstadt, German) with 1% inoculum concentration (V/V) for 24 h.

2.3. Antioxidant capacity

Lactococcus lactis subsp. *lactis* and ML23 (109 CFU/mL) were cultivated under different Cd stresses (0, 25, 50 and 100 mg/L) for 18 h, cell lysis was performed by ultrasonic (procedure: crush 5 s, interval 5 s, 30 cycles, power 200 W), centrifuged at 8000*g* for 10 min and the supernatant was collected for subsequent experiments.

Hydroxyl radical scavenging capacities were detected by Fenton reaction as described previously (Yan et al., 2009). Briefly, the supernatant was added into Fenton's reagent; the reaction was carried out at 37 °C for 60 min and monitored by measuring the absorbance at 536 nm with a UV–vis spectrophotometer (Hitachi U-1900, Japan).

The DPPH radical scavenging activities were detected as follows: DPPH (0.65 mM) was added into 60% ethanol/40% citric acid sodium citrate buffer (10 mM, pH 6) to make DPPH-ethanol solution. Then, 1 ml supernatant (as described above) was mixed with 4 ml DPPH-ethanol solution. The reaction was carried out at room temperature for 20 min. The decrease of DPPH radical concentration was measured by a UV–vis spectrophotometer (Hitachi U-1900, Japan) at 517 nm.

Superoxide dismutase (SOD) enzyme activity was determined by SOD Activity Assay Kit (BioVisioin, USA) and the reaction was measured by a microplate reader (Thermo Varioskan Flash, USA) at 505 nm. Glutathione (GSH) Elisa Kit (Abnova, USA) was used to detect the variation of GSH with cadmium addition at 12 and 18 h by a microplate reader (Thermo Varioskan Flash, USA) at 450 nm.

2.4. ATPase assays

The total ATPase and P-type ATPase (Ca^{2+}-ATPase and Na^+, K^+-ATPase) activities of cell free extract were performed by ATPase assay system kit (Innova Biosciences, USA). The reaction was carried out at room temperature for 30 min, and then measured by a microplate reader (Thermo Varioskan Flash, USA) at 630 nm.

2.5. Proteomics analysis

Lactococcus lactis subsp. *lactis* cultured in the presence and/or absence of cadmium (50 mg/L) were suspended in lysis buffer (7 mol/L carbamide, 2 mol/L thiocarbamide, 4% (W/V) CHAPS, 50 mmol/L DTT). The cells lysis was performed by ultrasonic and then digested with nucleic acid enzyme mixture (GE Healthcare, USA) for 1 h. The suspension was centrifuged at 25,000*g* for 15 min. The supernatant was collected and the concentration of protein was measured utilizing Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, USA).

2-DE for proteome was conducted according to previous study with a little changes (Jain and Bhatt, 2013). For isoelectric focusing (IEF), IPG strips (pH 4–7, 13 cm, GE Healthcare) were passively rehydrated for 14 h at 50 V and 20 °C with 100 μg protein extract suspended in 250 μL IEF buffer. IEF was conducted by a Multiphor III system (GE Healthcare, USA) at a current limit of 50 mA/strip at 20 °C. SDS-PAGE was performed in 12.5% gradient SDS-polyacrylamide gel. The proteins were visualized with Coomassie Brilliant Blue R-250 Dye. Image digitization was performed with an Image Scanner (GE Healthcare, USA) in transmission mode. Image Master 2D 7.0 software (GE Healthcare, USA) was used in gel analysis. All 2D experiments were carried out in triplicate.

A one way ANOVA test ($P < 0.05$) was used to select the significant differentially abundant spots between groups. For mass spectrometry (MS), spots picking of interest were conducted with preparative gels and subjected to in-gel trypsin digestion according to previous study (Sun et al., 2011) with minor modifications. The peptide mass spectra was obtained with a MALDI-TOF/TOF mass spectrometer (4800 Proteomics-Analyzer, Applied Biosystems, Foster City, CA, USA) according to method previously (Sun et al., 2007). GPS ExplorerTMsoftware 3.6 (Applied Biosystems, Foster City, CA, USA), MASCOT (Matrix Science, http://www.

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