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EVALUATION OF ANTIOXIDATIVE, PROTEOLYTIC, AND ACE INHIBITORY ACTIVITIES OF POTENTIAL PROBIOTIC LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL FERMENTED FOOD PRODUCTS

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Probiotic lactic acid bacteria (LAB) have been engrossed in plentiful food fermentations, known to man for millennia. The current investigation was aimed at investigating technical attributes, such as production of bioactive peptides, particularly ACE-I activity (anti-hypertensive property), proteolytic activity, and antioxidant activities of the potential probiotic LAB strains isolated from a diverse dairy and non-dairy based fermented foods. Among all ten LAB isolates, PFC21, isolated from sauerkraut, exhibited the highest antioxidative potential and showed maximum free radical scavenging ability using both ABTS (83.8±3.77%) and DPPH (59.4±2.18%) assays. It was followed by PD2 (*dosa* batter isolate) that showed (79.4±1.61%) activity in ABTS assay. PD2 revealed the highest proteolytic activity during 24 h and 48 h (with 0.82 and 1.12 absorbance, respectively) of fermentation at 37 °C; followed by a curd isolate, PC6, and PFC21 with 0.99 and 0.90 absorbance, respectively, at 48 h incubation. Furthermore, PD2 also showed the significantly ($P<0.05$) highest (49.39%) ACE inhibition followed by PFC21 (41.38%). These fascinating results led us to further evaluate the potential probiotic strains with regard to their utilization in the production of healthy quality foods with additional technical advantages.

Keywords: probiotics, antioxidative potential, bioactive peptide, angiotensin-converting-enzyme (ACE) inhibition

The growing attention to understand the role of food in human health has budged it from its primary role as a source of energy to the subtle action of biologically dynamic food components for wellbeing. Henceforth, there is a growing demand for functional foods at present. In India, variety of dairy and non-dairy fermented foods are known and consumed by large population as a part of their daily diet, but their probiotic role had not been investigated widely. Incorporation of probiotic microorganisms isolated from various regional fermented food products in market can positively enhance health status of larger segment of communities.

Reactive oxygen species (ROS) mediated oxidative damage of vital cell components is known to play crucial role in the development of chronic diseases such as diabetes mellitus, cancer, heart disease, Alzheimer, cataract, and aging (Mishra et al., 2015). It occurs as a result of imbalance between the generation of oxygen derived radicals and the antioxidant potential of the organism. Bioactive peptides produced from LAB through enzymatic hydrolysis and/or microbial proteolysis during milk fermentation are known to possess

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oxidative inhibitory capacity due to their ability to scavenge free radicals (Virtanen et al., 2007). Strains of LAB may reinforce the inherent cellular antioxidant defence by secretion of enzymes like superoxide dismutase (SOD) or promote production of the major non-enzymatic antioxidant and free radical scavenger glutathione (GSH). Currently, synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have limited use because of their suspected carcinogenic potential and thus, there is a shift towards the use of natural antioxidants (Chtourou et al., 2011). Recent studies suggest that probiotics may have a potential therapeutic role in ROS characterized gastrointestinal disorders (Spyropoulos et al., 2011).

Proteolysis is the most important biochemical process that occurs in cultured milk products during fermentation and storage; resulting in the release of bioactive peptides from specific amino acid sequences within the parent milk proteins that can provide physiological benefits (Yoshikawa et al., 2000). During fermentation, milk proteins get hydrolysed by LAB proteinases and peptidases resulting in an enhanced quantity of free amino groups and various forms of peptides (Kholif et al., 2011). Generally, the extent of proteolysis varies among strains and is time as well as strain dependent (Donkor et al., 2007).

Fermented milks are excellent sources of bioactive peptides owning specific physiological roles like antibacterial, anticancer, and antihypertensive activities. Among these, the antihypertensive peptides or Angiotensin converting enzyme inhibitors (ACE-I) are the most broadly studied. ACE inhibitory peptides have been isolated from a variety of fermented dairy products including cheeses, fermented milks, and yoghurts (FitzGerald & Meisel, 2000), and these bioactive peptides could serve as healthier and natural alternatives of ACE-I drugs (Donkor et al., 2007).

Strains of LAB used in current study have shown potential probiotic candidature in earlier investigations (Thakkar et al., 2015). Hence, in addition to the claimed health benefits of probiotics, the present investigation was principally aimed to determine the technical attributes of these potential probiotic LAB isolates, including production of bioactive peptides, particularly ACE-I activity, proteolytic activity, and antioxidant activities.

1. Materials and methods

1.1. Source of cultures

Total 10 test strains of LAB, i.e. PD2, PD11, PD30 (*Dosa* batter); PC6, PC27 (Curd); PH5 (*Handva* batter); PJ5, PJ29 (*Jilebi* batter); PSC6 (Soycurd); and PFC21 (Fermented cabbage) were transferred to MRS broth for propagation prior to experiments. The stock cultures were prepared in glycerol (80%) and preserved in –20 ºC. All microbiological media and chemicals were procured from HiMedia (Mumbai, India). These isolates were subjected to species level biochemical identification based on their carbohydrate fermentation profile using API test stripes (Table 1**)**.

1.2. Antioxidant activity

The antioxidant activity was evaluated by two different assays, ABTS and DPPH, making the use of cell free extract of overnight grown culture. BHT and vitamin C (ascorbic acid) were used as synthetic and natural standards (100 μg ml^{-1}).

Table 1. Details of selected LAB strains

LAB isolate	Isolation source	Carbohydrate fermentation (API Kit)	
		Species identity	% similarity
PD2	*Dosa* batter	*L. rhamnosus*	99.8
PD11	*Dosa* batter	*L. casei*	99.9
PD30	*Dosa* batter	*L. plantarum*	88.6
PC6	Curd	*L. casei*	99.8
PC27	Curd	*L. fermentum*	99.8
PH5	*Handva* batter	*L. fermentum*	97.4
PJ5	*Jilebi* batter	*E. faecium*	99.8
PJ29	*Jilebi* batter	*E. faecium*	95.9
PSC6	Soycurd	*L. rhamnosus*	99.9
PFC21	Fermented cabbage	*L. fermentum*	99.9

1.2.1. ABTS [2, 29-Azinobis (3-ethylene benzothiazoline) 6-sulphonic acid] assay. In this method, the total radical scavenging capacity is based on ability of a compound to scavenge the stable ABTS radical in 10 min (Re et al., 1999). The ABTS working solution was prepared by mixing 88 µl of 140 mM potassium persulphate with 5 ml of 7 mM ABTS stock solution followed by overnight incubation in dark bottles for generation of radicals. Then it was diluted with phosphate buffer saline (PBS) to adjust the absorbance at 734 nm to (0.7±0.02). An aliquot of 10 µl of product supernatant, collected after centrifugation at 10 000 *g* for 30 min, was transferred into 96 wells micro plate and to that 100 µl of ABTS in PBS solution was added and mixed for 10 sec. The decrease in the absorbance at 734 nm was recorded over the period of 10 min at 10 sec interval using Multiplate reader. The free radical scavenging activity (%) was calculated by the following equation; where 'blank' means ABTS solution and PBS without sample:

$$\% \text{ Scavenging activity} = \{(A_{734\ nm} \text{ blank} - A_{734\ nm} \text{ sample}) / (A_{734\ nm} \text{ blank})\} \times 100$$

1.2.2. DPPH [2,2 diphenyl-1-picrylhydrazyl] assay. The antioxidant activity from the extracts of products (fermented milk made from each isolate) was analysed through modified DPPH method (Hati et al., 2013). Hundred µl of the product supernatant from an appropriate dilution, collected after centrifugation at 10 000 *g* for 30 min, was loaded into 96 wells micro plate, mixed with 100 µl of freshly prepared DPPH solution, and incubated in dark for 120 min at 37 ºC after covering the micro plate with aluminium foil. The absorbance of the solution was measured at 517 nm against methanol using Multiplate reader. The experiment was performed in triplicates and the results were expressed as below; where 'blank' means DPPH solution and PBS without sample:

$$\% \text{ Scavenging activity} = \{(A_{515\ nm} \text{ blank} - A_{515\ nm} \text{ sample})/(A_{515\ nm} \text{ blank})\} \times 100$$

1.3. Proteolytic activity

The degree of proteolysis during the fermentation of milk was quantified by measuring free amino (NH2-) groups using *o*-phthalaldehyde (OPA) method (Donkor et al., 2005). Three ml curd samples were added to 3 ml of 1% (w/v) trichloroacetic acid (TCA). The suspension was vortexed and vacuum filtered using Whatman filter paper. One hundred and fifty microlitres

of TCA soluble peptides was added to 3 ml of OPA reagent, and after 2 min of incubation (20±1 °C), the absorbance was measured at 340 nm with using Systronic PC based double beam spectrophotometer (model:2202), India. The experimental procedure was repeated with untreated reconstituted skim milk (RSM) as a control. A relative degree of proteolysis was determined as the difference between the free amino groups in fermented milk and untreated milk with respect to their OD values.

1.4. ACE-inhibitory activity

ACE inhibitory activity was determined according to the technique of Cheung (1971) with a little modification (Papadimitriou et al., 2007). Active cultures were inoculated into a 10% reconstituted skim milk (at the rate of 2%), incubated for 24 h, and fermented milk was centrifuged at 10 000 *g* for 10 min at 4 °C (Eppendorf Centrifuge, US). The supernatant was collected and filtered through 0.2 μm cellulose acetate membrane filter. Thereafter, 50 μl of 5 mM HHL (hippuryl-L-histidyl-L-leucine) (10.74 mg HHL in 5 ml sodium borate buffer, pH 8.3) solution was mixed with 500 μl deionized water and 100 μl of sample (filtrate). The reaction was initiated by the addition of 20 μl (4 mU in 250 μl) of ACE enzyme and the mixture was incubated for 30 min at 37 °C. The reaction was terminated by the addition of 1000 μl of 1 M HCl. The hippuric acid liberated by the ACE was extracted with 1.7 ml ethyl acetate and then heated at 100 °C for 20 min in water bath. The residues containing hippuric acid were dissolved in 2 ml of deionized water and the absorbance of the solution was measured spectrophotometrically at 250 nm against blank. The extent of inhibition was calculated as follows.

$$\% \text{ ACE Inhibitory activity} = \{1–C–D)/(A–B)\}\times 100\ (\%)$$

where,

A: the absorbance of solution containing ACE but no sample

B: the absorbance of solution containing ACE but no sample and HCl

C: the absorbance of solution containing ACE, sample and HCl

D: the absorbance of solution containing ACE, sample but no HCl

The extent of inhibition is estimated as the concentration of the component that inhibits 50% of ACE activity (IC50) under the given conditions (Donkor et al., 2007).

1.5. Statistical analysis

The results of three individual experiments were gathered to generate the mean± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine the significance by using Minitab at $P<0.05$.

2. Results and discussion

India is a country of rich microbial diversity with an array of food habits. Fermented foods have been a regular part of meal for most Indians and have also been well known for its extended shelf life.

2.1. Antioxidant activity

Total antioxidative potential of ten lactobacilli cultures were determined by ABTS assay and DPPH assay; results were expressed in terms of percentage (%) activity (Table 2**)**. The

scavenging ability of the cell free extracts of the probiotic strains were compared with the standard antioxidants, ascorbic acid (vit. C), and BHT.

Table 2. Antioxidant activity of LAB isolates by ABTS and DPPH methods

Isolate no.	% Inhibition (24 h incubation)	
	ABTS method	DPPH method
PD2	79.4±1.61*a*	49.9±1.38*d*
PD11	42.1±2.72*d*	28.7±1.47*f*
PD30	60.9±1.83*c*	35.6±1.06*ef*
PC6	56.8±3.15*c*	41.7±1.18*e*
PC27	67.9±3.15*b*	51.5±3.89*d*
PH5	71.4±1.36*b*	46.5±3.10*de*
PJ5	43.0±2.24*d*	37.8±1.34*e*
PJ29	54.8±4.82*c*	31.3±1.39*f*
PSC6	73.4±1.82*b*	53.9±3.54*d*
PFC21	83.8±3.77*a*	59.4±2.18*c*
Standard BHT	84.5±2.54*a*	87.8±1.37*a*
Standard vit. C	83.7±2.48*a*	78.9±1.96*b*
	ANOVA table	
S.E.M.	2.01	1.62
F-test	*	*
CD	5.94	4.78
%CV	5.43	6.43

Values expressed are mean ± S.E.M; *a,b,c,d,e,f*: values with different superscripts differ significantly (P<0.05) in each rows & columns

Among all ten isolates, PFC21 (sauerkraut isolate) could be considered as the most appropriate for preparing fermented product with high antioxidative property, as it showed maximum free radical scavenging ability using both ABTS (83.8±3.77%) and DPPH (59.4±2.18%) assays followed by standards, vitamin C, and BHT. Similarly, PD2 (*dosa* batter isolate) displayed 79.4±1.61% scavenging activity with ABTS (non-significant with PFC21 and standards). Three isolates, i.e. PSC6 (53.9±3.54), PC27 (51.5±3.89) and PD2 (49.4±1.38), exhibited similar trend (non-significant to each other) as confirmed using DPPH free radical assay. All isolates exhibited >25% free radical scavenging activity following both methods; among all isolates, PFC21, PSC6, PD2, PC27, and PH5 showed very good antioxidant potential as compared to standards.

Hati and co-workers (2013) studied the antioxidative activity of probiotic lactobacilli in soy milk by ABTS method. *L. rhamnosus* C6 showed maximum antioxidative activity, i.e. percentage inhibition (97.0%), followed by *L. rhamnosus* NCDC 19 (92.0%), *L. casei* NCDC 17 (90.2%), *L. rhamnosus* C2 (89.1%), *L. rhamnosus* NCDC 24 (88.6%), and *L. casei* NCDC 297 (88.0%); besides, *L. rhamnosus* C6 strain also exhibited 50.2% inhibition using DPPH assay. Afify and co-workers (2012) reported scavenging potential of the cell free extracts

using ABTS assay; the maximum activity was observed with cell free extract of *Probionebacterium freudenreichii* (84.7%) followed by *L. rhamnosus* (84.6%) and *L. reuteri* (84.4%). Puniya and co-workers (2016) reported highest inhibition of 85.8% and 78.8% against ABTS radical action with LH16 (human isolate) and LM13 (dairy isolate). These differences could be due to different proteolytic activity of individual cultures, which results in a release of antioxidative peptides (Virtanen et al., 2007).

2.2. Proteolytic activity

The extent of proteolysis varied among strains with incubation time. PD2 liberated highest amount of amino acids during 24 h and 48 h (i.e. 0.82 and 1.12 OD values, respectively) of fermentation at 37 °C; followed by PC6 (0.99 OD) and PFC21 (0.90 OD) at 48 h incubation. For seven isolates, i.e. PD2, PD11, PC6, PC27, PH5, PSC6, and PFC21, the amount of liberated amino groups and peptides increased linearly till the end of fermentation (Table 3**)**. These findings are consistent with those reported by Leclerc and co-workers (2002) using *L. helveticus* strains. Except PD30, PJ5, and PJ29 isolates, the amount of liberated amino groups and peptides increased only slightly during fermentation from 0 to 12 h for other isolates, but from 12 to 24 h, these values increased significantly for all strains. Comparable growth pattern and proteolytic potential was observed by Donkor and co-workers (2007) while studying *L. acidophilus* L10, *L. acidophilus* La 4962, *B. lactis* B94, *B. longum* Bl536, *L. casei* L26, and *L. casei* Lc 279. These findings were consistent with those reported by Nielsen and co-workers (2001).

Table 3. Proteolytic activity of LAB isolates

LAB Isolates	Absorbance at 340 nm at incubation time (hours)				
	0	6	12	24	48
PD2	0.15±0.00*ab*	0.43±0.00*a*	0.53±0.02*a*	0.82±0.00*a*	1.12±0.00*a*
PD11	0.12±0.00*c*	0.27±0.02*bc*	0.40±0.01*d*	0.44±0.00*i*	0.68±0.00*h*
PD30	0.09±0.00*d*	0.16±0.02*d*	0.17±0.02*f*	0.65±0.00*i*	0.87±0.00*d*
PC6	0.14±0.00*b*	0.38±0.00*ab*	0.45±0.00*c*	0.59±0.00*f*	0.99±0.00*b*
PC27	0.16±0.00*a*	0.45±0.02*a*	0.51±0.00*b*	0.69±0.00*b*	0.86±0.00*e*
PH5	0.16±0.00*a*	0.4±0.01*a*	0.54±0.03*a*	0.54±0.00*h*	0.73±0.00*g*
PJ5	0.11±0.01*c*	0.22±0.04*c*	0.27±0.00*e*	0.44±0.00*i*	0.86±0.00*e*
PJ29	0.08±0.00*d*	0.25±0.02*c*	0.29±0.00*e*	0.58±0.00*g*	0.63±0.00*i*
PSC6	0.14±0.00*b*	0.33±0.03*b*	0.56±0.00*a*	0.63±0.00*e*	0.78±0.00*f*
PFC21	0.17±0.00*a*	0.36±0.01*b*	0.51±0.00*b*	0.66±0.00*c*	0.90±0.00*c*
	ANOVA table				
S.E.M.	0.00	0.02	0.01	0.00	0.00
F-test	*	*	*	*	*
CD	0.01	0.05	0.03	0.01	0.01
%CV	5.75	9.63	4.26	0.69	0.62

Values expressed are mean ± S.E.M; *a,b,c,d,e,f*: values with different superscripts differ significantly ($P<0.05$) in each rows & columns

The proteolytic pattern certainly had a strong effect on bacterial growth. In this context, PD30 (*dosa* batter isolate) exhibited very slow growth in the fermentative medium but still showed good proteolytic activity at 48 h of incubation; it was insignificant with fast growing culture PFC21 (fermented cabbage isolate). In a similar assay, *L. delbrueckii* ssp. *bulgaricus* Lb 1466 exhibited poor growth, though showed appreciable peptidase activity. It indicated that this organism might require some other growth factors in addition to free amino acids and peptides (Donkor et al., 2005). In another study, *L. rhamnosus* NS4 liberated the highest amount of amino acids during 24 h of fermentation at 37 °C (79.7% ACE inhibitory activity) followed by *L. delbrueckii* 009 (67.1%) due to their strong proteolytic systems compared to the other isolates (Hati et al., 2015).

2.3. ACE inhibitory activity

ACE-inhibitory peptides can be produced by enzymatic hydrolysis of the milk proteins during the fermentation of milk with specific strains of LAB having antihypertensive activity. We have attempted to isolate LAB strains having potential to exhibit ACE-inhibition (Table 4**)**. PD2 strain exhibited significantly ($P<0.05$) the highest (49.4%) ACE inhibition followed by PFC21 (41.4%). PC27 (37.1%) and PSC6 (33.8%) isolates showed almost similar ACE inhibitory activity ($P>0.05$). All other isolates were found to exhibit ACE inhibitory profile ranging from 29.3–12.6%. The lowest inhibition was reported for PD30 (12.6%).

Table 4. ACE inhibition (%) by LAB isolates in skim milk

LAB isolates	ACE inhibition (%)
PD2	49.4±1.47*a*
PD11	23.9±0.87*de*
PD30	12.6±0.62*g*
PC6	26.4±1.73*d*
PC27	37.1±1.47*c*
PH5	29.3±2.31*d*
PJ5	18.0±0.35*f*
PJ29	21.2±2.09*e*
PSC6	33.8±2.28*c*
PFC21	41.4±1.07*b*
ANOVA table	
S.E.M.	1.11
F-test	*
CD	3.28
%CV	5.58

Values expressed are mean ± S.E.M; *a,b,c,d,e,f*: values with different superscripts differ significantly ($P<0.05$) in each rows

Two strains, *L. rhamnosus* NS4 and *L. bulgaricus* 009, gave maximum ACE inhibitory activity, i.e. 79.7% and 67.1%, respectively, compared to other isolates (Solanki, 2014). ACE inhibition profile of *L. helveticus* strain was evaluated in three media spectrophotometrically, where the lowest ACE inhibitory activity was obtained for WPI-enriched milk (2.24–3.51 mg ml^{-1}) hydrolysis (Yamamoto et al., 1994). Higher ACE inhibitory activity was measured

for skim milk medium (1.15–1.68 mg ml^{-1}), whereas caseinate enriched milk provided the highest ACE inhibitory activity (0.6–1.1 mg ml^{-1}). Results indicate that proteolysis by starter bacteria is indispensable to generate ACE inhibitors in fermented milks. Also, the nature of protein substrate used in the medium was found to be more important in the production of ACE inhibitors than the degree of protein.

3. Conclusions

Traditional fermented foods had emerged as rich sources of probiotic LAB. Outcomes of the present work indicate that LAB strains isolated from traditional Indian fermented foods showed excellent antioxidative potential, proteolytic and antihypertensive attributes in addition to previously proven probiotic potential. Starters with such vital characteristics can help to develop functional probiotic food with a wide range of health benefits. However, it warrants further in vitro and in vivo studies to elucidate the potential therapeutic benefits of isolated lactic acid bacteria strains.

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