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RESEARCH ARTICLE

DETERMINATION OF ANTIOXIDANT PROPERTIES OF *LACTOBACILLUS* SPECIES FROM DIFFERENT SOURCES

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ABSTRACT

Lactic acid is found to be one of the most industrially essential acids that have a wide spread application such as food preservative, curing agent and flavouring agent. Lactic acid bacteria has antioxidant activity which is associated with multiple health protective effects such as prevention and treatment of diarrheal disease, inflammatory bowel disease, immunomodulation, treatment of cholesterolemia, prevention of systemic infections, prevention and treatment of allergies, and remedy for lactose intolerance. This investigation was designed to analyze the antioxidant capacity of lactic acid bacteria in order to provide characteristic parameter and theoretical basis for its further utilization as a natural antioxidant. In this study, different food samples were used to isolate lactic acid bacteria (LAB). Bacterial isolates that showed catalase negative, gram positive, glucose positive, mannitol positive and lactose positive were selected for further studies. The antioxidant activity of cells and different cellular fractions of *Lactobacillus* sp was evaluated by reducing power, total antioxidant activity, ferrous ion chelator, flavonoid test, scavenging DPPH and total phenolic content present. Further to confirm HPLC and TLC were also done. The probiotic potential of the isolates was found out by checking their pH and bile tolerance ability. All the isolates shown growth at pH 4. The study highlights a simple and rapid method for identification of *Lactobacillus* sp. On the other hand, in presented study media optimization was also done to maximize the production of LAB.

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INTRODUCTION

Free radicals are highly reactive chemicals that have the potential to harm cells. They are formed when an atom or a molecule either gains or loses an electron. It plays an important role in many normal cellular processes. They can be hazardous to the body and damage all major components of cell at high concentration (VilasraoJ.Kadam, 2010). Most common type of free radicals that contain the element oxygen are produced in living tissue. It is also called as "reactive oxygen species" or ROS (VilasraoJ.Kadam, 2010). Oxidation is important for most of the organisms for the production of energy to carry out biological processes (Jan-Ying Yeh, 2011). The uncontrolled production of oxygen derived from free radicals is involved in many disease such as cancer, rheumatoid arthritis, heart disease, ageing, Parkinson's disease (Jan-Ying Yeh,2011). Antioxidants interact with and neutralize free radicals, thus preventing them from causing damage (Maryam A.S.Abubakr, 2012). Endogenous antioxidant are produced by body and are used to neutralize free radicals. The antioxidants are called endogenous or exogenous. These exogenous antioxidants are also called as dietary antioxidants. Vegetables, Fruits and grains are rich sources of dietary antioxidant. Antioxidant based drugs for the treatment of complex diseases have attracted research interest in natural antioxidant. Probiotic should be screened for their antioxidant potential. Probiotics are live microorganisms that beneficially affect the health of the host's by improving microbial balance (Kahouli, 2015). Study on lactic acid bacteria shows that implementation of probiotic strain increases health benefits, including improvement in the nutritional value of food, controlling gastrointestinal infection, improving digestion of lactose, controlling serum cholesterol level and controlling some type of cancer (She-Ching Wu, 2011). LAB strains are used as probiotics as they are resistant to host. Lactobacillus is the predominant genus of lactic acid bacteria found in fermented sourdough, whey from fermented milk, yogurt, fruits like apple, strawberry, whey containing viable lactobacilli have been used as a prophylactic aid and treatment of intestine by humans.

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The purpose of the investigation was to determine antioxidant property of lactic acid bacteria isolated from different food samples by using of DPPH radicals, ferrous chelating activity (FCA), reducing power and HPLC of cell free extract of probiotic strains.

MATERIALS AND METHODS

Chemicals and Reagent: Chemicals such as 1,1 –diphenyl2-picryl hydrazyl (DPPH), ferrozine, ferrous chloride, Folin-Ciocalteu's reagent, aluminium nitrate $Al(NO_3)_3$, potassium acetate CH_3COOK, sodium carbonate solution, sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4), Ascorbic acid and Ninhydrin were used.

Isolation of LAB: Lactic acid bacteria were isolated from different sources like sourdough of grain wheat and bajara, apple, yogurt with blueberry, whey from fermented milk according to the standard methodology described by Maryam A.S Abubakr, 2012. The sample was spread plated on de Man Rogosa and Sharpe (MRS) agar plate and were incubated at 37^0 C for 48 hrs. The isolates were tested for catalase activity by using the 4% (v/v) hydrogen peroxidase solution. The presence of catalase in the cell was indicated by formation of bubbles. Then the isolates which were catalase negative were selected. All the bacterial isolates were maintained in glycerol stock and stored at -4^0C for further studies.

Morphological Biochemical characterization: Morphological and biochemical characterization of the organismswas done according to the standard methodology described by Bergeys's Manual of Determinative Bacteriology (Holt *et al*., 1984).

Optimization of the Production Medium: (JatindraNath Mohanty, 2014): The two different production medium were optimized by changing their carbon and nitrogen source for getting the optimum production of crude lactic acid.

Optimization of Carbon and Nitrogen Source for MRS Broth: In MRS broth sucrose and fructose were used as a carbon source in place of glucose and checked for optimum production. Yeast extract and Beef extract were used as a nitrogen source in place of Meat extract and were checked for optimum production at 10g/L.

Optimization of Carbon and Nitrogen Source for Skimmed Milk Medium: In Skimmed Milk Broth sucrose and fructose were taken in place of glucose as the source of carbon to check for optimum production whereas Beef extract and tryptoneis taken as nitrogen source in the place of yeast extract and checked for optimum production.

Titrimetric assay (Titratable Acidity) of Lactic acid: (Jatindra Nath Mohanty, 2014)

This method takes into account the concentration of dissociated hydrogen molecules and undissociated hydrogen ions. Acidity is related to hydrogen in the solution so TA to measure the total acidity is indication of lactic acid level. In this process the sample is titrated against 0.1N NaOH with the addition of 2-3 drops of phenolphthalein indicator until the colour of the sample turns light pink. The percentage of purity of lactic acid is calculated using the given formula –

$$R = \frac{V(titr) \times C(titr) \times Mw. \times F \times 100}{1000 \times W(smp)}$$

Where, R = % of lactic acid

V (titre) = Total volume of titrant needed to reach the end point in ml
C (titre) = concentration of titrant
Mw. = molecular weight of lactic acid =90.08
F = Dilution factor
W (smp) = sample amount in either gram or ml.
100 is multiplied to obtain the percentage of lactic acid present.

Separation and determination of organic acid using Thin Layer Chromatography: (Ki-Yong Lee, 2001)

By thin layer chromatography the presence of lactic acid is being confirmed. In this process the sample is run on the glass slab containing a layer of silica gel in a TLC chamber containing the desired solvent. The percentage composition of the solvent system was chloroform/ methanol /glacial acetic acid (65:15:2) (v/v). To ensure sufficient supply of solvent vapour the spotted TLC plate eas placed at the bottom of chromatographic chamber. After closing the chamber development of chromatogram was allowed to proceed till the solvent front reached $3/4^{th}$ of the total area.TLC plate was air dried and plate was sprayed with Ninhydrin indicator solution (Ninhydrin- 0.1g, acetic acid – 0.5ml, acetone 100 ml). Colour was developed by heating (1-3 min) in a hot dry oven and Rf value was calculated.

Cell free culture supernatant preparation

The bacteria were cultured in 50 ml of MRS Broth medium at 37ºC for 48hrs kept in shaker. Cells were collected by centrifugation at 10,000 (rpm) for 8 minute, 25º C. Cell free extract wes collected and used for the further measurement of antioxidant activity.

Determination of total antioxidant capacity

Determination of T-AOC (Total Antioxidant capacity): (Dachang Wu, 2014)

Many antioxidants in organism can make Fe^{3+}reduce into Fe^{2+},which can combine with ferrozine to form solid chelation showing various colours. Based on this theory, antioxidant ability can be determined by chemical colorimetry. In this work the total antioxidant capacity was determined using ferrozine. Initiation of the reaction was done by the addition of 0.2 ml of ferrozine into the cell free extract, vortexed thoroughly and the absorbance was taken at 520nm against blank. The results were expressed as unit per milligram sample protein. T-AOC was calculated according to the following equation;

Total antioxidant activity(%) =

$(A_{520\ nm}$ sample $/A_{520nm}$ control) X 100%

Reducing power activity: (Jan-Ying Yeh, 2011)

The reducing power assay determines the ability of antioxidant to donate electron using potassium ferricyanide reduction

method. The reducing capacity of a compound may serve as an indicator of its potential antioxidant activity. Sample under test (0.5ml of cell free extract, celllysate) was mixed with 0.5ml of 0.2M sodium phosphate buffer (pH 6.6) and 0.5 ml of 1%potassium ferricyanide (w/v) and the resultant mixture was cooled rapidly after incubation at 50^0C for 20min. The mixture was then centrifuged at 3000 rpm for 5 min after adding 0.5ml of 10% trichloroacetic acid (w/v). The supernatant was (1ml) was mixed uniformly with 1ml of 0.1 % ferric chloride (w/v) till 10 min and the absorbance taken at 700nm was measured with blank of distilled water. Higher absorbance indicated higher reducing power.

DPPH free radical –scavenging activity: (Jan-Ying Yeh, 2011)

DPPH radicals are widely used for the evaluation of antioxidant activities of proton donating substances according to their hydrogen donating ability. DPPH radicals accept electrons or hydrogen radicals to form stable diamagnetic molecules. A volume of 5ml of DPPH in methanol (0.005 DPPH in 50 ml of methanol) was added to 1ml of the cell free extract, mixed vigouresly and allowed to stand in the incubator for 90 minutes at room temperature. The absorbance was measured at 517nm. Methanol was used as a blank, while DPPH solution in methanol served as the control. DPPH solution in ascorbic acid was used as a standard. The radical scavenging activity of the samples was expressed as percentage inhibition of DPPH absorbance.

$$Inhibition(\%)=[(abs_{control}-abs_{test})/abs_{control}]X100$$

Where, $abs_{control}$= absorbance of the control sample (DDPH solution without cell free extract)
Abs_{test}=absorbance of the test sample(DPPH solution with cell free extract)

Ferrous chelating activity (FCA): (MaryamA.S.Abubakr, 2012)

The ability of different peptides generated by *Lactobacilli* to chelate ferrous ions was assessed using the method of Decker and Welch. The concentration of the cell free extract (5-100µg/ml) was added to 0.1 ml solution of 2mM ferrous chloride ($FeCl_2$). Then the reaction was initiated by adding 0.2 ml of 5mM ferrozine. The mixture was shaken vigorously and kept at room temperature for 10min. Absorbance of the solution was measured at 562 nm in spectrometer after the mixture was equilibrated. The Fe^{+2} chelating ability of extract was monitored by measuring the ferrous ion- ferrozine complex. EDTA was used as control. The percentage of inhibition of ferrozine –Fe^{2+}complex formation was given by the following formula:

$$\text{Ferrous ion chelating ability (\%)} = [(A_O - A)/A_0] \text{ X } 100$$

Where, Ao is the absorbance of the control solution (containing all reagent except extract); A is the absorbance in the presence of the sample.

Total Phenolic content and flavonoids: (Samuel Lallianrawna, 2013)

Total phenolic content was determined with Folin-Ciocalteu method. The Folin–Ciocalteu (F–C) reagent is sensitive to reducing compounds and polyphenols. A blue coloured complex is produced. The F-C assay relies on the transfer of reducing equivalents (electrons), in the alkaline medium, from phenolic compounds to phospho-molybdic/phosphotungstic acid complexes. The blue coloured complexes manifested are determined on a UV-visible spectrophotometer by monitoring the absorbance at 765 nm. The reference compound used for comparison was galic acid. The values are evaluated as the mg equivalent of gallic acid per g of extract. Mixture of 0.1 g of extract, 0.8 ml of deionised water and 0.1 ml of Folin-Ciocalteu reagent was incubated at room temperature for 3 min. After adding 0.3 ml of Na2CO3 (20% w/v), the mixture was incubated at room temperature for 30 min. To obtain a calibration curve, various concentrations of gallic acid solutions (0.08, 0.07, 0.06, 0.05, 0.04, 0.03, 0.02, 0.01mg/ml) were prepared. Appropriate volume of sodium carbonate solution was added in each flask and the final volume was adjusted with distilled water. Measurements were carried out after 1 h at 765 nm on a UV-visible spectrometer against the reagent blank. The calibration curve of concentration against the absorbance was plotted. 1 mL of stock solution of extracts was transferred in a 25 ml flask; similar procedure was adopted for the preparation of calibration curve. With the help of the calibration curve, the phenolic concentration of extracts was determined. Total flavonoid content was determined by modified colorimetric method. 1.5 ml of sample was taken and 75 µl of 5% $NaNO_2$ solution was added. After 6 min, 150 µl of 10% AlCl3.6H2O was added to the mixture, which was kept at room temperature for 6 minutes, followed by the addition of 0.5 ml of 1M NaOH and the total volume was made up to 2.5 ml with the addition of deionised water. The resulting solution was mixed well and the absorbance was measured at 510 nm on a UV-VIS spectrophotometer. For the blank, the samples were replaced with an equal volume of deionised water. A standard calibration curve was prepared with 0.01, 0.05, 0.1, 0.2, 0.4,0.6,0.8mg/ml of quercetin (in deionised water). The total flavonoid content was expressed as the mg equivalents of quercetin (QE) per g of sample.

High Pressure liquid chromatography (HPLC): (Dan Cristian VONDAR, 2010)

HPLC was performed on Hitachi liquid chromatography consisting of a model L-6200 pump and a model L-4200 UV-Vis detector set at 320nm. The cell free extract were used and the mobile phase which was used is methanol solution using an isocratic elution with a flow rate of 0.6ml/min. The calculation of lactic acid was made from the peak area registered at specific retention time for lactic acid.

RESULTS

Isolation of LAB

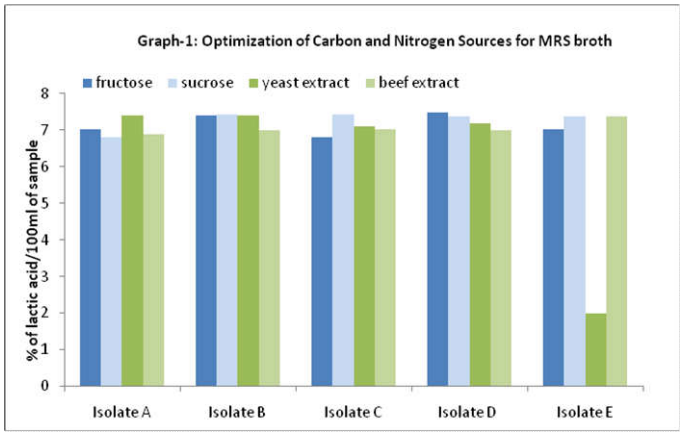
In the present study total 5 samples were used, total 8 bacteria were isolated. 5 were found to be Gram positive and 3 were found to be Gram negative (Table-1).

Table 1. Total Number of bacteria obtained from different sample sources

No	Sample	Total bacteria
1	Sour dough of wheat	2
2	Bajra	2
3	Apple	1
4	Yogurt	1
5	Whey	2

Optimization of the production medium: (JatindraNath Mohanty, 2014)

High amount of lactic acid was obtained in MRS broth as compared to Skimmed milk broth. When different nitrogen and carbon sources were added in MRS media, it was found that maximum lactic acid was obtained in beef extract N source. On the other hand, maximum lactic acid was obtained in sucrose C source Graph-1.

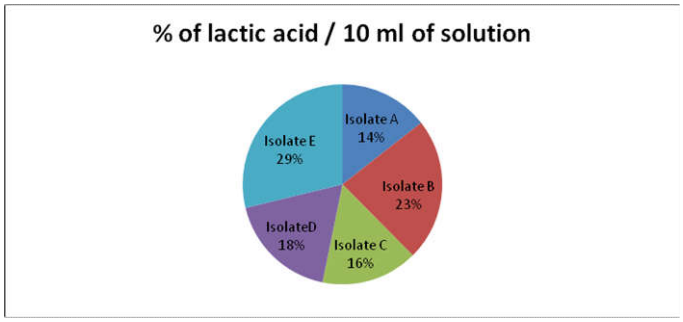


Titrimetric assay of lactic acid: (JatindraNath Mohanty, 2014)

The percentages of lactic acid were calculated by using the titrimetric method of the isolates. The maximum percentage of lactic acid was showingby this method in isolate D followed by isolate B. Table-2.

Table 2. Percentage of lactic acid per 10ml of solution

No	Samples	Consumed NaOH during titration (ml)	% of lactic acid / 10 ml of solution
1	Isolate A	4	3.6
2	Isolate B	6.5	5.8
3	Isolate C	4.4	3.9
4	Isolate D	5	4.5
5	Isolate E	8	7.2

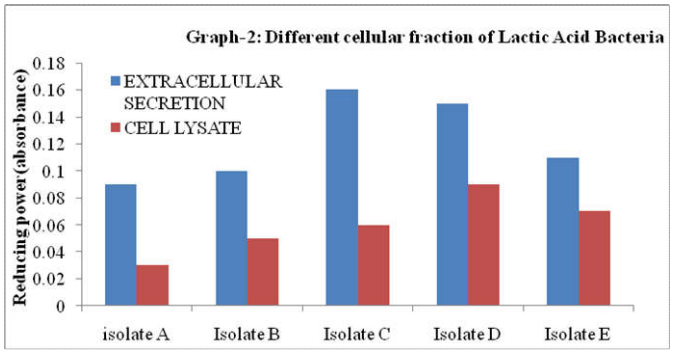


Graphical view of % of LAB Isolated from different food sample.

Antioxidant activity:

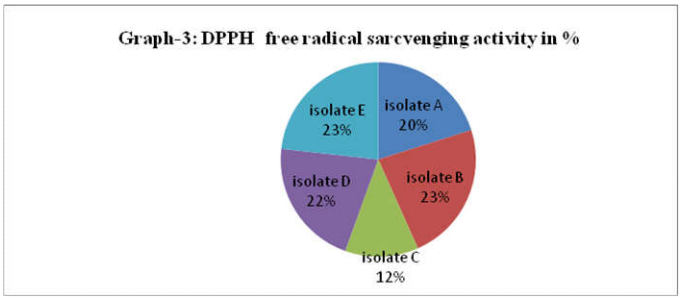
Reducing power : (Jan-Ying Yeh,2011)

The reducing power assays determine the ability of electron donation of antioxidants using the potassium ferricyanide reduction method. The reduction of the Fe^{3+}/ferricyanide complex to the ferrous form is caused due to reducers in the test solution. The reducing power of extracellular secretion was the highest, cell lysate take the second place. Graph-2:



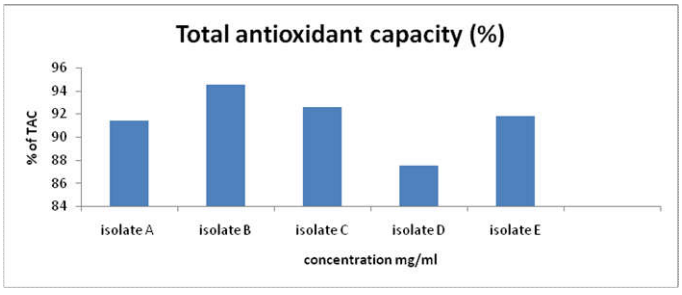
Scavenging of 2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical: (Jan-Ying Yeh, 2011)

DPPH, a relatively stable organic radical has been widely used in the determination of antioxidant activity of cell free extract of the bacteria. The highest antioxidant activity was observed with isolate B and isolate E i.e. 43.21%. The DPPH values of standard ascorbic acid are 69.52%. The isolate B and E represents the highest antioxidant activity Graph-3.



Total antioxidant capacity: (Dachang Wu, 2014)

Determination of the level of the total antioxidant capacity has significant importance. The total antioxidant capacity of isolates ranges from 87.54-94.55% of standard ascorbic acid at concentration ranging from 0.1 to 1 mg/ml. Results showed that the isolate B and isolate E had highest total antioxidant capacity.



Ferrous chelating ion (FCA): (Maryam A.S.Abubakr,2012)
Free ferrous iron is sensitive to oxygen and gives rise to ferric iron and superoxide and forms hydrogen peroxide. Reaction of ferrous ion with hydrogen generates the hydroxyl radical, which oxidizes the surrounding bio molecules. In this process, known as the fentonreaction, hydroxyl radical production is directly related to the concentration of copper or iron. To compare the ferrous chelating activity of isolates, ethylene diamine tetraceticacid (EDTA) was used as a positive control. The FCA values were in the range of (62.11-54.03%) for 24

hours, respectively. However, FCA values decreases significantly with longer fermentation time.

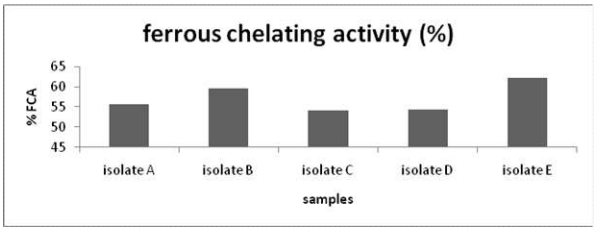


Fig.4. Ferrous chelating activity of LAB isolates

Flavonoid and Phenoliccontent: (Samuel Lallianrawna, 2013)

Total phenolic content

The concentration-absorbance calibration curve for 8 stock standards of gallic acid solution is given in the fig 5. The measured absorbance value at 765 nm for the indicated concentration of gallic acid solutions in the range of 0.2-1.39. Within this range of concentration (10-80mg/ml), the calibration curve of gallic acid clearly exhibited linearity. i.e. isolate E is showing highest phenol content followed by isolate B by extrapolating values in standard graph.

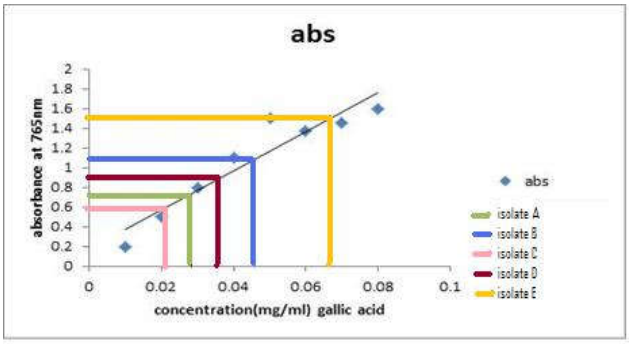


Fig. 5. Standard curve of Gallic acid

Total flavonoid content

The calibration curve for 6 sequentially and independently prepared stock standard solutions of quercetin that depicts the concentration of quercetin against the absorbance, as presented in fig 6. The absorbance value increased proportionally upon increasing the concentration of quercetin from 0.05 to 0.8mg/ml. A slight deviation from the linearity seemingly occurred at the higher concentration region of quercetin calibration plot. Total flavonoid concentrations of the isolates were obtained highest to lowest by extrapolating values in standard graph of quercetin.

HPLC (High Pressure Liquid Chromatography): (Dan Cristian VONDAR, 2010)

A study has demonstrated that isolates from a fermented sample contain a large amount of antioxidant, and these components may reduce oxidative damage via free radical-scavenging activity. It has been reported that lactobacillus contain various antioxidants such as gallicacid, quecetin etc. The changes in the major antioxidative component in lactobacillus during development are unknown; hence the level of several organic acid (acetic acid) and phenolic compounds (quercetin and gallicacid) were investigated. HPLC of the isolates and ascorbic acid as standard were done. The chromatograms of isolates were showing peaks which comes under the standard ascorbic acid. From this study it was observed that isolates may have lactic acid.

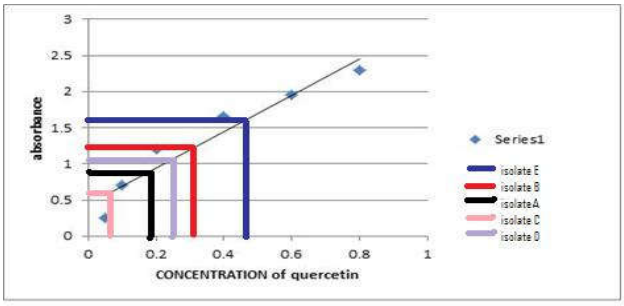


Fig. 6. Standard curve of quercetin

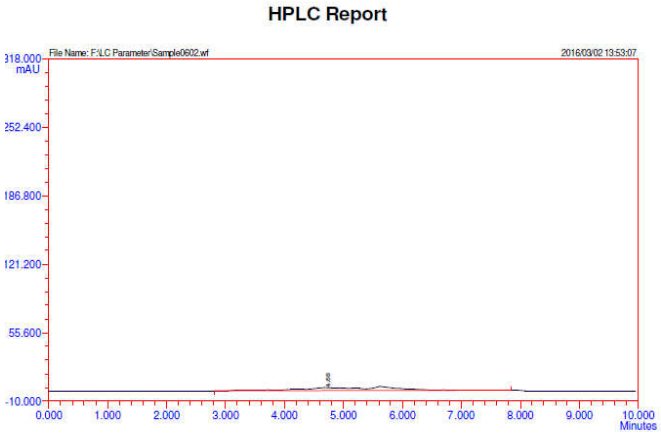


Fig. 7. HPLC chromatogram of fermented sample A

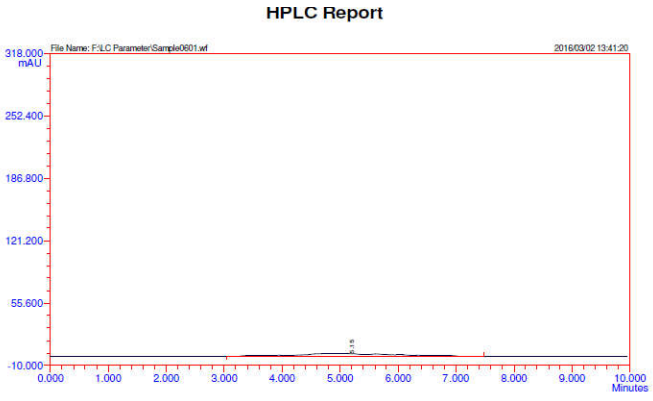


Fig. 8. HPLC chromatogram of fermented sample B

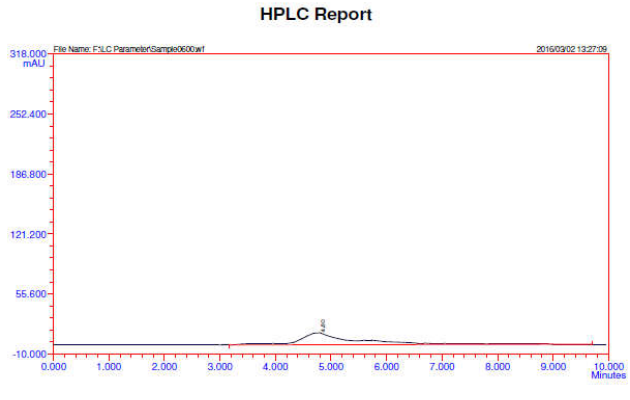


Fig. 9. HPLC chromatogram of fermented sample C

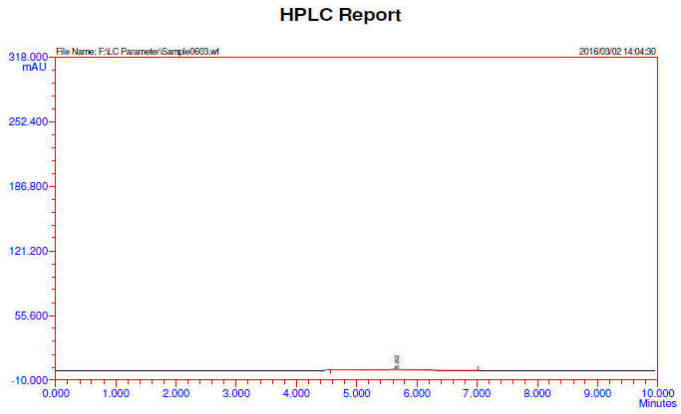


Fig. 10. HPLC chromatogram of fermented sample D

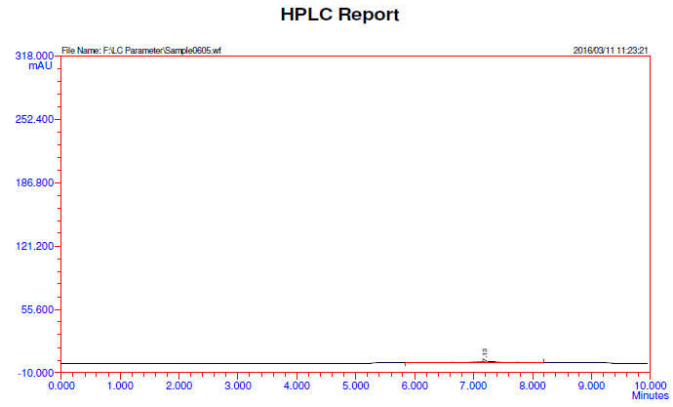


Fig. 11. HPLC chromatogram of fermented E

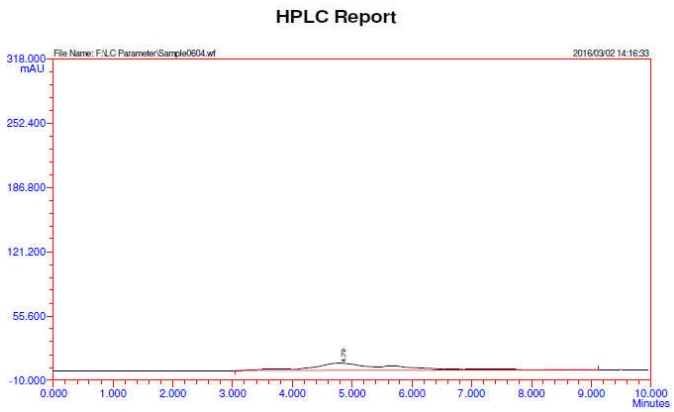


Fig. 12. HPLC chromatogram of ascorbic acid

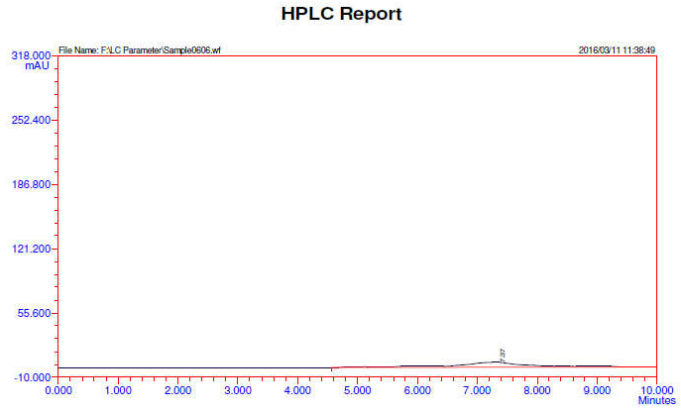


Fig. 13. HPLC chromatogram of strawberry sample

DISCUSSION

Natural defence mechanism eliminate negative effects of the activity of free radicals. However they may not be always adequate to totally neutralize all endogenous and exogenous free radicals. In this study, the antioxidant activity of lactobacillus bacteria from different sources were obtained (AkinniyiOsuntoki, 2010). The reducing capacity of a compound may serve as a indicator of its potential antioxidant activity. Recently it has been reported that there is a direct correlation between antioxidant capacities and reducing power. DPPH is commonly used to determine radical scavenging activity of natural compounds as it is a stable free radical (Md.Delowar Hossain, 2013). The scavenging ability of cell free extract of the probiotic strain was compared with the standard antioxidant ascorbic acid. From the above results we could conclude that the LAB have antioxidant activity and the value depends mainly on the way of antioxidant determination and type of bacteria. Antioxidative ability, the most important organism member of defense system, is closely related with health. Many factors such as hunger, trace element absorptive power, age and hormone level etc, influence the function of body's defense system. This hypofunction often lead to various diseases. It is easy to cause inflammation, cancer, diabetes, immune system and other diseases when the body's total antioxidant capacity is reduced (Dachang Wu, 2014). Chelating power measures the effectiveness of compounds to compete with ferrozine for ferrous ion. High chelating power extract reduces the free ferrous ion concentration by forming a stable iron chelate. The extent of Fenton reaction is decreasesd which is implicated in many diseases. The chelating effects of various extracts on Fe^{2+} were determined by the formation of ferrozine Fe^{2+}complxes. Chelating agents are able to capture ferrous ion before ferrozine, thus hindering the formation of ferrozine-Fe^{2+} (Jan Ying Yeh, 2011). Our findings showed that the isolates have high potential for FCA. Phenolic compound are a class of antioxidant agents which act as free radical terminator and their bioactivities may be related to their abilities to chelate metals, inhibit lipoxygenase (Ibrahim, 2014). The polyphenols compounds have exhibited inhibitory effects on mutagenesis and carcinogenesis in humans (Rajeshwari Sahu, 2013). The effect of phenols on cell proliferation and cytotoxicity is largely investigated and debated aiming at explaining the potential protective role towards oxidative stresses and tumor development (Jose Antonia Curiel, 2015). Total phenol concentration of the isolates were obtained are highest to lowest. The flavonoid and phenolic contents were quantified of the isolates. Many studies have reported that the levels of many compounds, such as beta-carotene, polyphenols and flavonoids are increased because of fermentation by lactic acid bacteria (She-Ching Wu, 2011). A study has demonstrated that isolates from a fermented sample contain a large amount of antioxidant, and these components may reduce oxidative damage via free radical- scavenging activity. It has been reported that lactobacillus contain various antioxidants such as gallic acid, quecetin etc. The changes in the major antioxidative component in lactobacillus during development are unknown; hence the level of several organic acid (acetic acid) and phenolic compounds (quercetin and gallicacid) were investigated (Dan Cristian VONDAR, 2010).

Conclusion

The development of food with beneficial effects in addition to the provision of nutrients is a growing niche. The aim of the

study is to focused on antioxidant activity of LAB strain obtained from different food samples. The isolate B and isolate E of food sample i.e., bajra and whey possess good properties and showed highest activity. The whey of fermented skim milk has the potential to be a good dietary supplement into food formulation or cosmetics for prevention of oxidative stress related diseases such as coronary heart disease, atherosclerosis and cancer. Probiotic have been widely assessed for their effect on lipid profiles such as total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides as well as liver and kidney function. The study also provide the characteristic parameters and theoretical basis for its further utilization as a natural antioxidant.

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