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SHORT COMMUNICATION

Evaluation of lactase activity in probiotic cultures containing inulin rich plant extracts as prebiotic sources

Rimsha Akhtar, Daheem Azhar, Sundus Mubashir and Shahnaz Choudhry*

Department of Biotechnology Kinnaird College for Women, 93 Jail Road, Lahore 54000, Pakistan.
Email: shahnaz.ch@kinnaird.edu.pk

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ABSTRACT

Aims: The present study deals with the isolation and identification of lactase producing probiotic strains from camel and sheep milk, determination of the enzyme activity by β-galactosidase assay (Miller Assay) in the presence of garlic, peas, onion and leeks extracts containing inulin as a prebiotic component.
Methodology and results: The two isolates were screened for lactase producing ability to degrade lactose on MRS agar at 37 °C. These were identified as *Lactococcus lactis* from camel (Marecha) milk and *Lactobacillus casei* from sheep (Kajli) milk through morphological and biochemical tests using MRS medium. The optimized pH and temperature of both strains were 6 and 35 °C, respectively. Among the three concentrations used (0.2%, 0.4%, 0.8%), the optimal concentration of inulin rich onion and leeks extracts was 0.8% for maximum growth of *L. casei* and of the peas extract for *L. lactis* growth. 0.2% garlic extract was more effective prebiotic source for *L. lactis* growth. 0.8% commercial inulin used as a positive control was less effective as compared to plant extracts used in the study. With o-nitrophenyl-β-D-galactoside) used as a substrate in the enzyme assay, maximum lactase activity obtained with 0.8% concentration of garlic extract is 7.10 Miller Units as compared to the peas extract with 6.17 Miller Units from *L. lactis. Lactobacillus casei* has produced more lactase, 6.85 Miller units with onion extract than with leeks extract, 6.43 Miller Units. Pure commercial inulin used as a control has given maximum enzyme activity as 9.14 Miller Units at 0.2% concentration.
Conclusion, significance and impact of the study: It is concluded that the extracted prebiotic may enhance lactase activity of the probiotics to supplement the development of food products for lactose intolerant patients.

Keywords: Lactobacillus casei, *Lactococcus lactis*, inulin, lactase activity, β-galactosidase assay

INTRODUCTION

Live microbial food supplements known as probiotics have the ability to enhance growth and development of other organisms (Macfarlane *et al*., 1999; Ananya, 2014). They are non-toxic, non-pathogenic and are able to survive in the gastrointestinal tract. *Lactobacillus* (*L. casei*, *L. acidophilus, L. lactis*) and *Bifidobacterium* (*B. longum, B. breve, B. infantis*) are commonly known probiotics present in dairy products. Permanent survival of live microbes is difficult in intestine, so they need to be digested on regular basis to promote health metabolism and improve symptoms of lactose intolerance. Probiotic strains are used to improve lactase enzyme activity. These are commonly present in products like yogurt, cheeses, fruits, vegetables, soil and in some supplements. Besides lactic acid bacteria, *Saccharomyces, E. coli*, *Enterococcus*, *Streptococcus* and *Bacillus* species behave as probiotics. Probiotics are responsible for performing dual functions as they act as microbial agent for fermentation of food products and are responsible for imparting health benefits (Welch *et al*., 2011).

Prebiotics are defined as non-digestible component that beneficially affect the host organism. These are the dietary substances that act as the food sources for friendly gut microorganisms to promote their growth instead of the harmful bacteria (Coxam, 2007). Oligosaccharides and polysaccharides have prebiotic activity and stimulate the growth of *Lactobacillus* and *Bifidobacterium* in colon of humans. Leeks, onions, asparagus, oats, wheat, garlic and chicory roots are natural sources of prebiotics. Inulin is a fructan, a well-known prebiotic linked with gastrointestinal tolerance because it has low level of digestible carbohydrates and provide immunity by boosting up white blood cells (Roberfroid, 2007). Inulin is not only low calorie but also has a role as a prebiotic in the regulation of intestinal

***Corresponding author**

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flora. Inulin can also be used as a fat or sugar substitution ingredient. Food and pharmaceutical industries are using inulin in the making of functional foods, nutritional composites and medicines. It acts in the organism in a similar way as dietary fiber, contributing to the improvement of the gastrointestinal system conditions (Roberfroid *et al.*, 1993).

Lactase (β-galactosidase) is a hydrolytic enzyme that has the capability of performing two activities, one is the breakdown of lactose into glucose and galactose and second is the breakdown of β-glycosides, but in majority of the cases low activity of lactase become the cause of digestive insufficiency, the condition known as lactose intolerance (Mozumder *et al*., 2012). Large populations of people suffer from lactose intolerance due to lactase enzyme deficiency. So, there is a need of looking for the best possible probiotic and prebiotic combinations for enhanced lactase activity followed by the development of food products supplemented with these effective combinations and their production by food industries. Such products are easy to digest and absorb due to breakdown of lactose by probiotic lactase activity. This will facilitate digestion and absorption in lactose intolerant individuals with gastrointestinal disorders. The purpose of this study was to isolate and identify probiotic strains one each from fresh camel and sheep milk considered to be a rich source of probiotics, prepare inulin rich garlic, peas, onion and leeks extracts as prebiotic sources, and to check the effectiveness of the probiotic-prebiotic combinations for bacterial lactase activity by β-galactosidase assay.

MATERIALS AND METHODS

Sample collection

Ten milliliter of fresh raw milk samples each from camel and sheep commonly known as Marecha and Kajli were collected in sterilized falcon tubes from a village near Lahore and Sabzazaar colony, Lahore, Pakistan. These were stored in refrigerator at 4 °C.

Isolation and identification of bacterial strains

Both milk samples were streaked on MRS agar (agar 15 g, ammonium citrate 2 g, beef extract 10 g, K_2HPO_4 2 g, glucose 20 g, $MgSO_4$ 0.1 g, $MnSO_4$ 0.05 g, peptone 10 g, Polysorbate 80 (Tween 80) 1 mL, CH_3COONa 5 g, yeast extract 5 g in autoclaved distilled water up to 1 L) and incubated overnight at 37 °C. Primary and secondary cultures of the isolates were prepared in MRS broth. These were then screened for lactase enzyme activity on MRS agar containing 50 µL X-gal in 50 mL medium (0.1 %) as a substrate. Presence of blue colonies after incubation indicates active production of lactase enzyme (Aitken, 2012).

Identification of strains was based on Gram staining, motility test, biochemical tests including catalase test, carbohydrate fermentation test and oxidase test and growth of cultures at different temperatures and pH (Perloff *et al*., 1990; Zourari *et al*., 1992; Hedberg *et al*., 2008; Forouhandeh *et al*., 2010).

Preparation of inulin rich garlic, peas, onion and leeks extracts

Sun dried samples of garlic, peas, onion and leeks were ground in autoclaved distilled water, filtered and centrifuged at 6000 rpm for 20 min. To 5 mL of clear supernatant 20 mL concentrated sulphuric acid was added drop wise. Deep wine color indicates the presence of inulin (Nair and Surendran, 2005). Temperature and pH were optimized to get maximum growth in MRS broth as an absorbance at 600 nm of the bacterial isolates identified as *L. lactis* from camel milk and *L. casei* from sheep milk. Different concentrations (0.2%, 0.4%, 0.8%) of clear inulin rich vegetable extracts, and commercial inulin used as a positive control were optimized to get maximum bacterial growth at 540 nm. Garlic and peas extracts were added as substrates to *L. lactis* culture, whereas, onion and leeks extracts were used for *L. casei.* Optimization of prebiotic concentration was carried out in triplicates in Phenol Red Lactose broth (protease peptone 10 g, beef extract 1 g, NaCl 5 g, lactose 5 g, Phenol red 0.018 g in distilled water per litre). Standard deviation was applied to values obtained from triplicate experiments.

β-galactosidase assay

Beta-galactosidase (lactase) enzyme activity was measured by o-nitrophenyl β-D-galactopyranoside (ONPG-β-Gal) assay also referred as Miller assay (Zhang and Bremer, 1995). Absorbance of 48 h bacterial culture was taken at A_{600nm}. 1 mL of each culture was transferred to eppendorf tube and centrifuged at 12,000 rpm for 5 min. Supernatant was discarded and cell pellet was suspended in 80 µL of permeabilization solution A (100 mM sodium hydrogen phosphate (Na_2HPO_4, 20 mM KCl, 2 mM $MgSO_4$, 0.8 mg/mL CTAB, 0.4 mg/mL sodium deoxycholate, 5.4 µL/mL beta-mercapto-ethanol) and incubated for 30 min at 37 °C temperature. After incubation, 600 µL of substrate solution B (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg/mL o-nitrophenyl-β-D-Galactoside (ONPG), 2.7 µL/mL β-mercaptoethanol) was added to each sample and incubated again for 1 h at 37 °C until yellow color appeared. Then, 800 µL of 1M Na_2CO_3 was added to stop the reaction. Absorbance was measured at A_{420}. Lactase enzyme activity was calculated in Miller Units defined by the formula below:

$$1 \text{ Miller Unit} = 1000 \times A_{420} - (1.75 \times A_{550}) / (t \times v \times A_{600})$$

Where, A_{420} is o-nitrophenol absorbance, A_{550} is the absorbance cell debris multiplied with 1.75, t is the time of reaction in minutes, v is the volume in mL, and A_{600} is the absorbance of cell culture.

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RESULTS AND DISCUSSION

Identification of bacterial isolates

Screening test

In this study, the probiotic isolates, *L. casei* from sheep milk and *L. Lactis* from camel milk produced blue colored colonies on MRS agar at 37 °C indicating the production of lactase enzyme (Figure 1). Sheep milk is abundant with lactic acid bacteria with the range of 10^2-10^6 CFU/mL. According to an approach, the range of *L. lactis* in sheep milk is 10^9 CFU/mL .Camel milk is also dominated by mesophilic bacteria like *Lactobacillus species* with the range between 10^{2}-10^7 CFU/mL. De Man, Rogosa and Sharpe (MRS) medium was used to isolate, cultivate probiotic strains. This medium has the capability of supporting the growth of all *Lactobacillus* and *Lactococcus* bacteria from different sources like oral cavity, dairy products which includes milk, yogurt and foods (MacFaddin, 1985). In x-gal staining also known as Lac Z staining, lactase is responsible for hydrolysis of x-gal, an organic lactose molecule analog into galactose and 5-bromo-4-chloro-3-hydroxyindole molecule. This second molecule generated is oxidized into 5, 5–dibromo-4, 4–dichloro-indigo, an insoluble blue end product which indicates the presence of Lac Z gene in lactase positive species (Burn, 2012).

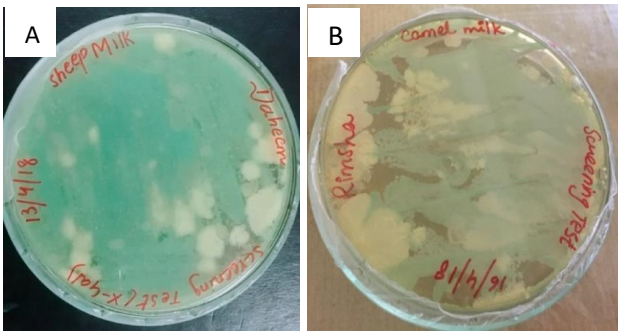

Figure 1: Blue colonies of lactase producing (A) *L. casei* (sheep milk) and (B) *L. Lactis* (camel milk) on MRS agar plates infused with x-gal.

Morphological and biochemical tests

The two isolates were Gram positive purple color rods and cocci and proved to be non-motile as their growth was confined to stab line. *Lactobacillus* species are Gram positive rods and cocci as well as they are non-motile. Lactic acid bacteria isolated from camel milk have been reported as non-motile. *L. lactis* and *L. casei* both showed negative catalase test and positive carbohydrate fermentation test. *L. casei* and *L. lactis* were catalase negative and these genera lack catalase enzyme as indicated by the absence of bubble formation after the addition of hydrogen peroxide (Ahmed and Kanwal, 2004; Goyal *et al*., 2012). Oxidase test was negative for *L. lactis*. Phenol red lactose broth (PRLB) is the differential media for variety of microbial species. As the color and pH of medium changes, pungent and foul odor produces due to the production of mixed acids, especially butyric acids. Carbohydrate fermentation test involves phenol red lactose broth for fermentation of lactose into simple sugar molecules with change in color of PRLB from red to yellow and the pH of the medium to acidic (Vera, 1950). Positive carbohydrate fermentation test confirms lactase production by these two strains.

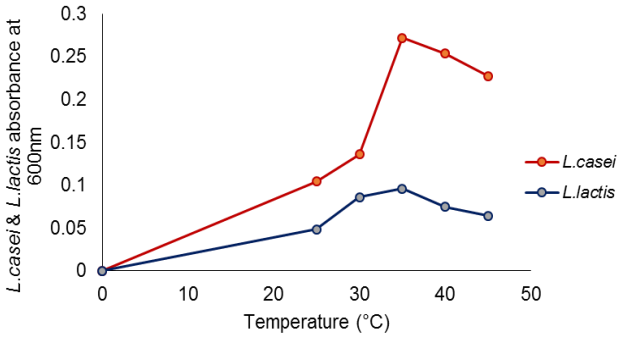

Figure 2: Effect of temperature on *L. casei* and *L. lactis* growth.

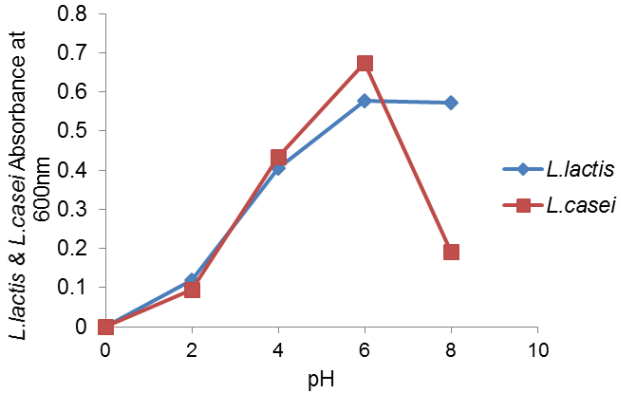

Figure 3: Effect of pH on *L. lactis* and *L. casei* growth.

Effect of temperature, pH and prebiotic concentration

Maximum growth of *L. lactis* and *L. casei* was obtained at 35 °C and pH 6 shown in Figure 2 and Figure 3. Tables 1 and 2 show the effect of prebiotic concentration on probiotic growth. 0.8% inulin rich leeks extract is more effective in enhancing *L. casei* growth as compared to the same concentration of onion extract. For *L. lactis* growth, 0.8% peas extract, and 0.2% garlic extract are more effective. However, 0.2% commercial inulin (chicory root) has shown to be more effective for growth of both the species. According to a study, the estimated amount of inulin in leeks and onion were 3-10% and 2-6%, respectively, whereas, garlic bulb contains 9-16% inulin content in it (De Souza *et al*., 2012). Despite the lesser

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Table 1: Influence of prebiotic concentration on *L. casei* growth.

Strain	Substrate	Prebiotic concentrations (%)	Mean values of absorbance with standard deviation*
Lactobacillus casei	Leeks extract	0.2	0.4638±0.0741
		0.4	0.4646±0.0247
		0.8	0.4921±0.0365
	Onion extract	0.2	0.3515±0.0138
		0.4	0.3905±0.0350
		0.8	0.4037±0.0249
	Commercial inulin	0.2	0.4312±0.0299
		0.4	0.4191±0.069
		0.8	0.3671±0.055

*Mean values with standard deviation for experiments performed in triplicates.

Table 2: Influence of prebiotic concentration on *L. lactis* growth.

Strain	Substrate	Prebiotic concentrations (%)	Mean values of absorbance with standard deviation*
Lactococcus lactis	Garlic extract	0.2	1.4082±0.3846
		0.4	1.2344±0.2703
		0.8	1.2289±0.2660
	Peas extract	0.2	0.8601±0.0915
		0.4	0.9254±0.0904
		0.8	0.9373±0.1649
	Commercial inulin	0.2	0.5433±0.018
		0.4	0.5078±0.0075
		0.8	0.4895±0.0880

*Mean values with standard deviation for experiments performed in triplicates.

inulin content of onion reported, lactase activity obtained from *L. casei* with 0.8% onion extracts is more than leeks extracts used in this study. The usefulness of onion is due to the phytochemicals and flavonoids present in it. Flavonoids increase the quercetin aglycone concentration. LPH-lactase acts on flavonoids and quercetin that help in reduction of hyperglyceamia in lactose intolerant individuals (Ranjbar, 2017).

Peas contain least amount of inulin then all other sources but they have a wide range of health benefits due to the presence of phytochemicals responsible for many antinutritive factors, starch, fibre, vitamin and mineral content. Polyphenolics present in peas have antioxidant and anticarcinogenic activity. One of the beneficial prebiotic effects of galactose oligosaccharides is enhancement of lactase enzyme production (Dahl *et al*., 2012). Least enzyme activity has been obtained with peas in our study in *L. lactis.*

Lactase activity through β- Galactosidase assay

It was observed that 0.8% concentration of onion extract with *L. casei* shows higher lactase activity (6.85 Miller units) as compared to the same concentration of leeks extracts (6.43 Miller units). Lactase from *L. lactis* shows higher enzyme activity (7.10 Miller Units) with 0.8% concentration of garlic extracts as compared to 0.8% concentration of peas extracts (6.17 Miller Units). As the prebiotic concentration increases from 0.2% to 0.8% in both the probiotic cultures, lactase enzyme activity also increases. The enzyme activity with 0.2% commercial inulin from Chicory root was maximum (9.14 Miller units) as compared to the same concentration of the plant extracts. This activity gradually dropped beyond 0.2%. Inulin majorly acts as an inducer for increased enzyme activity. In case of plant extracts, it has been observed that more prebiotic concentration (0.8%) is required for enhanced enzyme activity because these extracts in crude form besides having inulin as the major component have other components which may interfere with lactase activity. With purified inulin from the selected plant sources, results would have been different. Thus, it was observed that lactase activity was highest with 0.8% concentration of inulin rich plants extracts. According to (Miremadi and Shah, 2012), inulin stimulates the overall metabolism of lactic acid bacteria, in general, the levels of all metabolic end products increased in the presence of inulin used as the sole energy source. Error bars in Figures 4 and 5 indicate standard deviation for beta-galactosidase assay performed in triplicates. As the values of standard deviation were very less, little variance was found in inulin rich plant extracts and commercial inulin.

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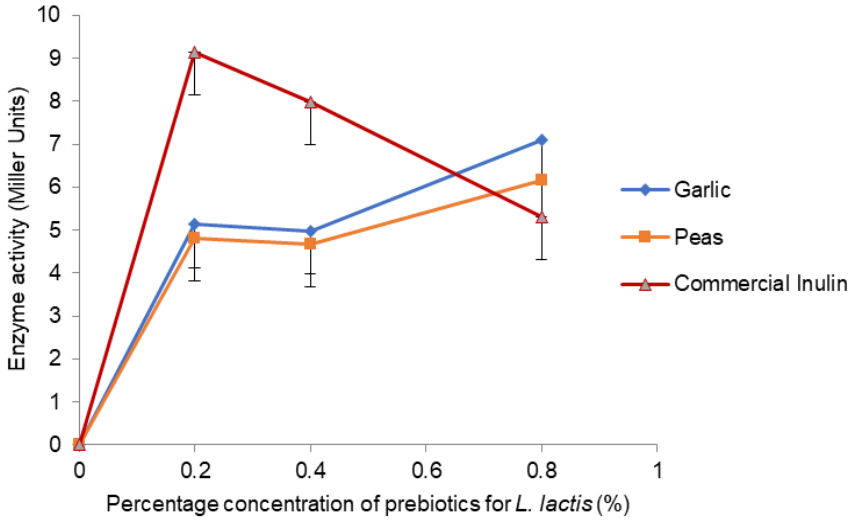

Figure 4: Effect of inulin containing garlic and peas extracts, and commercial inulin on lactase activity through β-galactosidase assay (Miller Assay) for *L. lactis*.

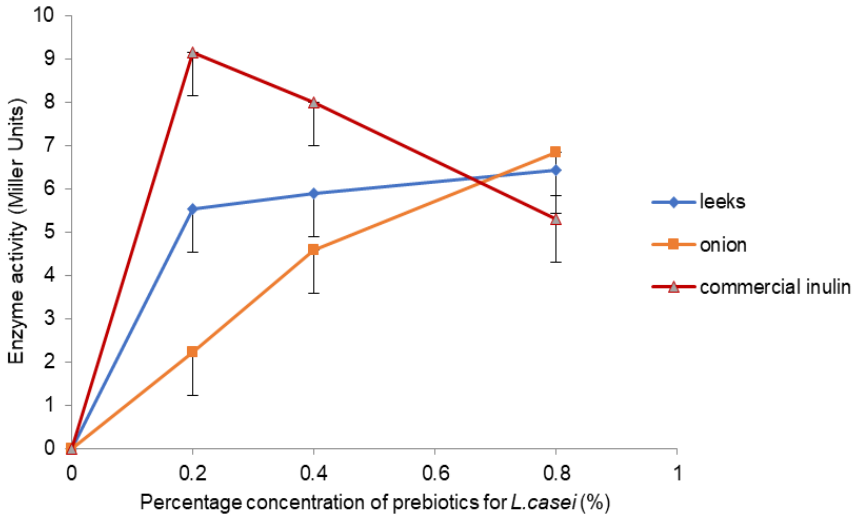

Figure 5: Effect of inulin containing leeks and onion extracts, and commercial inulin on crude lactase activity through β-galactosidase assay for *L. casei*.

CONCLUSION

Lactococcus lactis and *L. casei* were isolated from camel and sheep milk, as lactase positive probiotic bacterial strains. Inulin containing extracts prepared from garlic and peas for *L. lactis,* from onion and leeks for *L. casei* have proved to be effective substrates for bacterial growth and lactase enzyme activity. Inulin rich 0.8% onion extract with *L. casei* gives higher lactase activity (6.85 Miller units) as compared to same concentration of leeks extract (6.43 Miller units). Similarly, 0.8% concentration of garlic extracts with *L. lactis* gave maximum enzyme activity (7.10 Miller units) as compared to the same concentration of peas extracts (6.17 Miller units). Commercial inulin being a purified product shows maximum lactase activity with 0.2% concentration.

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