Study of fermentation of pulses for nutritional benefits using *Lactobacillus* spp.

Abstract

Pulses are an excellent source of carbohydrates and other nutrients. Many traditional fermented foods are prepared from cereals and combinations of cereals and pulses that usually contain LAB, *Bacillus, Enterococcus* and yeast. *Lactobacillus* can be used as a starter culture for such fermentation using pulses, as very few reports are available on fermented pulse-based products. Hence, pulses dal flour was used as a source for isolation of *Lactobacillus* to maintain their functionality, growth characteristics and activity during food processing. In this study, we investigated the potential of lactobacilli from fermented pulses in reducing carbohydrates, ability to degrade non-digestible oligosaccharides, production of α-galactosidase enzyme, optimizing production of lactic acid and change in heat-stable anti-nutritional factors. *Lactobacillus* isolated from different pulse dal flour, exhibited excellent growth during fermentation in MRS-glucose medium. The nature of growth curve for these strains in MRS-glucose medium was described using a modified Gompertz equation and showed shortest latency phase in MRS-glucose medium. All the lactobacilli performed very well in terms of survival efficiency in modified-MRS (mMRS) containing sucrose, sucrose + starch, raffinose, raffinose + starch based mediums. Among *Lactobacillus* species only *Lb. brevis* displayed the highest α-galactosidase activity, where raffinose was added as the sole carbohydrate source in the medium. The isolate was further tested in pigeon pea fermentation, where it showed maximum activity and complete hydrolysis of non-digestible oligosaccharides was observed. Further, screening for maximum lactic acid production was carried out using a simple synthetic medium consisting of pulses, yeast extract, and manganese sulphate as a carbon, nitrogen and mineral source, respectively. The maximum lactic acid obtained in optimized pulses fermentation; while, acetic acid was not detected in any samples. Finally, effect of lactobacilli on anti-nutritional factors was determined in pulses bean fermentation. This research showed that lactobacilli were able to ferment soaked red lentil, black gram, faba beans, pigeon pea beans and lower the tannin and saponin content significantly. Fermentation lowered these anti-nutrients better when inoculated with *Lb. plantarum, Lb. pentosus, Lb. plantarum* and *Lb. brevis*. Overall, usage of these Lactobacilli isolates could be an excellent opportunity to design and develop a novel pulse-based fermented product contributing to beneficial bioactive compounds and improve properties of food.

Introduction

Pulses belonging to the *Leguminosae* family; *Leguminosae* are the most important group of the *Dicotyledonae*, being one of the largest families of flowering plants with 18,000 species and, the second worldwide food crops, after cereals. Although pulses belong to the traditional dietary habit in many countries, their consumption gained popularity throughout the world due to the consumers awareness of the nutritional and functional values. Pulses are rich in carbohydrates and protein and comprises of basic nutritive constituents including dietary fibers, minerals, vitamins, amino acids, fatty acids and phytochemicals (1). Pulses contribute in treating several health associated diseases, but the main drawback limiting their consumption is the presence of high levels of α-galactosides, mainly raffinose.

α- Galactosides are carbohydrates localized particularly in seeds and different tissues in plants**.** α- Galactosides comprises sugars that have galactose units, linked to the glucose moiety of sucrose as α-1, 6-galactosyl residue at the end. Humans lack in producing α- galactosidase enzyme (α- Gal). These oligosaccharides are further metabolized by microflora, yielding considerable amounts of carbon dioxide, methane and hydrogen gas in the large intestine, ultimately leading to disturbance in abdominal pain, flatulence, diarrhea, nausea, *etc* (2). Therefore, to overcome these problems and enhance the nutritional value of a food product; attempts have been made over the years to eliminate these α-galactosides from pulses. Thus, the use of bacteria producing α-galactosidase offers a promising solution for the degradation of this oligosaccharide, during fermentation using *Lactobacillus* (3). *Lactobacillus* species are Gram positive, catalase negative, strictly hetero-fermentative, producing lactic acid, acetic acid and CO_2 as end products, and non-spore forming bacteria. They are found in variety of environment including fermented cereals, vegetables, fish, *idli* batter, and yogurt. *Lactobacillus* fall in the category of generally recognized as safe (GRAS) organisms. At present, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus helveticus*, *Lactobacillus acidophilus* and *Lactobacillus brevis* are capable of hydrolyzing α- galactosides into palatable form (4). These enzymes are either intracellular or extracellular in most organisms and produce enzymes with α-D- galactopyranosyl groups supplemented as carbon sources in the medium. Although extracellular α- galactosidase enzymes are advantageous and show good yield and stability than intracellular enzymes (2). For the elimination of these galactosides,

fermentation is one such conventional method that helps in improving physicochemical and functional properties of fermented food.

Fermented foods designated as “naturally fortified functional food” are considered as most superior functional foods for humans. Due to their high nutritional properties, they help in maintaining healthy intestinal microbiota, manage physiological homeostasis of body and protect against various diseases. These days, pulse-based fermented products are gaining more attention worldwide for their involvement in mineral and phenol bioavaibility, protein digestibility and degradation of anti-nutritional factors like α-galactosides (raffinose, stachyose), *etc*. Anti-nutritional factors also known as anti-nutrients are poisonous substances that can be found in most food and able to limit the nutrient available to the body. Lactobacilli have been implicated in the lowering of anti-nutrients and unwanted materials in food during fermentation with many cerals and pulses based food networks. In addition, scientists conducting different research stated that lactic acid fermentation, such as that used with lactobacilli, is the safest way to preserve food. There are credible literatures showing fermented food as the most important part in human diet and increasing demand for incorporation of live lactobacilli because of their beneficial role in preventing diseases as well as enhancing food safety by killing food-borne pathogens, thereby providing stability and long shelf life to food. Hence, these strains are implemented during fermentation to make the product economically attractive.

Rationale

Various fermented foods are popularly prepared in India and other parts of the world but very few pulse-based products exist currently in the market, fermented preparation using *Lactobacillus* are mostly prepared from milk, vegetables, fruits, sourdough, cereals (mixture of cereals with pulses). This study was undertaken for developing fermented pulse-based food product using *Lactobacillus*; hence, pulse split beans flour were used as a source for the isolation of lactobacilli to maintain their functionality, growth characteristics and activity during food processing. These lactobacilli act as a carrier in pulses and serves food of high calorie, improved functionality and sensory qualities. Very few reports were available till date (april, 2021) on isolating lactobacilli as possible candidates from pulse-based fermented foods. Moreover, a recent study by Lavanya et al., (2021) reported isolation of *L. plantarum* from fermented *Jangri*, a delicious flower shaped Indian sweet prepared by deep frying of batter. *Jangri* batter is made up of black gram (without skin) after soaking in water for 3–4 h (5). However, studies on

isolation of lactobacilli from fermented pigeon pea, lima bean, red lentils, and green gram are not done so far, but reports are available with *Lactobacillus* as a starter culture for fermentation of soy products and fermentation of pigeon pea using combination of *Bacillus* and *Lactobacillus* sp. (6) but not with other pulses. Studies on α- galactosidase from *Lactobacillus* during fermentation of pulses enable in understanding feasibility of decrease in α- galactosides in pulses, that are believed to cause disturbance in humans after eating. There is report available containing presence of intracellular enzyme from *Lb. brevis* but to the best of my knowledge no reports still exist claiming the presence of extracellular α-gal enzyme from *Lb. brevis* (3). Fermentation has lead to decrease in other anti-nutritional factors.

Objectives

1. Isolation, identification and characterization of *Lactobacillus* from fermented pulses flour.
2. Study of change in carbohydrates and α- galactosides in fermented pulse beans and study of α galactosidase from selected *Lactobacillus*.
3. Study of change in other anti-nutritional factors during fermentation of pulses for nutritional benefits.

Materials and method

Different pulses dal (green gram, pigeon pea, chickpea, faba bean, black gram, red lentil flour) was purchased from a local market in Vadodara. In a sterile container, flour was mixed with sterile deionized water and batters were kept at incubation to obtain lactobacilli. Identification of *Lactobacillus* at species level was done according to Bergey's Manual of Determinative Bacteriology. Further, phenotypic characterization was done on the basis of growth at different temperatures, pH values and NaCl concentrations. For genotyping characterization of isolates, samples were sent to Labreq Bioscientific for genomic DNA isolation and 16s rRNA sequencing. The evaluation of lactic acid production from glucose using MRS medium was conducted for the isolated LABs. The growth kinetics of experimental data using MRS-glucose was fitted to modified Gompertz equation. Further, single and combination of carbohydrates like sucrose, soluble starch + sucrose, raffinose, and raffinose + soluble starch were selected to check utilization of carbon source in m-MRS medium. The experiment was conducted in modified medium and components were suspended separately in different flasks. Samples were withdrawn at different time intervals for monitoring microbial counts, pH drop, decrease in sugars, lactic acid *etc*.

The isolates were evaluated for the production of α- galactosidase enzyme in a medium containing pNPG. Simultaneously, the effect of pH on enzymes was determined using different pH and temperature ranges. Similarly, growth medium composition was evaluated for the production of α-gal activity using different carbon sources like raffinose, starch and sucrose. Finally, to check the behavior of pulses, beans were soaked in water and fermentation was performed. Fermented pulses were sampled to check for microbial population, decrease in pH, α-gal activity, hydrolysis in raffinose and sucrose using thin layer chromatography, and lactic acid.

To enhance production of lactic acid, a medium was prepared using pulse beans. 20 g of whole pigeon pea (PP), red lentil (RL), black gram (BG), and broad bean (LB) beans were weighed and thoroughly washed and soaked in water for 6-7 h. Next day peas (without skin) were minced and final volume was made up to 100 ml using water. Then, in a test tube, 20 g of yeast extract and 0.05 g $MnSO_4$ were autoclaved separately. Add components in sterile condition and mix thoroughly. The mixture was used as production medium or with the nutrient supplement of MRS medium, pH, lactic acid and cell growth were noted every 24 h at 37 ˚C. Samples were centrifuged at 10,000 rpm for 10 min and further analyzed. Further, lactic acid was detected using HPLC and calculation of lactic acid was made from the peak area registered at specific retention time for lactic acid, and considering the regression curve factor. Similarly, analysis of ethanol and acetic acid was performed using a GC-FID.

Study of heat- stable anti-nutritional factors in pulses was carried using different processing methods like soaking, dry roasting, dehulling and fermentation. ANFs like saponin and tannin were determined at 560 and 700 nm using a UV–VIS spectrophotometer.

Results

The first aim was to carry out flour batter fermentation for the isolation of *Lactobacillus*. During the initial hours of fermentation, no increase in volume was detected, and pH of batter remained same, as time progressed, subsequent rise in volume of batter was also observed. The rise in the batter volume can be linearly linked to the leavening process which takes place due to involvement of *Bacillus*, *Lactobacillus*, and *Weissella* spp. A total of eight bacterial isolates were obtained from flour batters based on their cell morphology. These isolates showed resistance to temperature, saline stress and acid tolerance. Based on the morphological, physiological characteristics and identification of all these four isolates using 16S rRNA sequencing method, the presumed organisms were of *Lactobacillus* and *Weissella* species and exhibit 99% to 100%

similarity. A phylogenetic tree was generated using Neighbor Joining method by BLAST program to show the relationship between isolated and reference strains. Some *Lactobacillus* species exhibited the closest similarity while some shared a long distanced relationship with other isolates.

M1- *Lactobacillus plantarum* (MK530231), U1 *Lactobacillus pentosus* (MK530234), VIP1 *Lactobacillus plantarum* (MK530235), TIP1 *Lactobacillus brevis* (MK530232), Mu1 *Weissella jogaejeotgali*, T1 *Weissella hellenica* (MK530233), C1 *Weissella paramesenteroides* (MK530237), V1 *Weissella confuse* (MK530236).

Pulses foods are rich in proteins and consist of 25 % to 65 % of total carbohydrates like mono- and oligosaccharides and some polysaccharides; while starch is the most abundant followed by sucrose, raffinose, stachyose and verbascose (7). The carbohydrate composition of pulse seed usually contains fewer amounts of monosaccharides and disaccharides such as sucrose (1-3%) slightly higher in amount. The concentration of sucrose varies among species in pulses, reportedly up to 7 % in soybean varieties. Based on the composition of carbohydrate in pulse seeds, media containing glucose were tested for growth kinetics and lactic acid production.

In this study, the growth kinetics of species was investigated using modified Gompertz equation derived by, Zwietering *et al*. to describe the behavior of growth of an organism following the exponential law. A clear lag phase was observed for three hours after which the bacteria started to grow exponentially for all the species. This might be due to the lower initial bacterial count that caused increase in lag phase of bacterial growth. The main factor influencing the growth during lag phase is the inoculum size and the change in the physiological environment of original and new growth medium.

Further, the acidification was calculated using modified Gompertz equation and the results showed similar behavior in MRS-glucose medium producing higher lactic acid (15.40 g/l) from glucose as substrate. These strains exhibited shortest lag phase and longest stationary phase with highest biomass increment in *L. pentosus* and *W. paramesenteroides*. The maximum production of lactic acid in this experiment was obtained by *L. pentosus* and *W. jogaejeotgali*; however, the concentration of lactic acid in *W. paramesenteroides* was comparatively higher at 36 h which declined at 48 h. This change in concentration indicates depletion of carbon source in medium, thereafter the organism starts to consume lactic acid. On the other hand, decrease in cell growth was found in both the medium on 48 h by *Weissella* strains that might be due to the accumulation

of lactate resulting in growth inhibition. Moreover, this inhibition occurs due to change in potential that breaks cell membrane of microorganism, the accumulation of anions or due to acidification in cytosol.

All the five isolates grew well in m-MRS containing sucrose, sucrose + starch, raffinose and raffinose + starch medium. The obtained lactobacilli can be tagged as amylolytic due to their ability to metabolize starch during fermentation, indicating the degradation of starch as fermentation time progresses. Another parameter for utilization of sugar was sucrose that was possible because of the presence of invertase enzyme in an organism that has the ability to break down sucrose to glucose and fructose for catabolism. Additionally, utilization of raffinose and raffinose + starch by lactobacilli was simultaneous and achieved the same viable cell count for all the isolates when both sugars were present in the medium.

All *Lactobacillus* isolates and a reference strain were evaluated for the production of α-gal activity and cell growth. TIP1 culture showed the highest α-gal activity (1.30 U/ml) compared to other isolates. The extracellular fraction had considerably higher enzyme activity than the intracellular fraction. Although, it is an advantage that this enzyme is extracellular, as it provides high yield, stability and pH range (2-11) (6.5) in activity compared to intracellular enzymes. These enzymes showed good temperature stability (38℃), while loss in activity was observed at higher temperature. Similarly, maximum enzyme production was reported in acidic condition. Further the effect of different carbon source was examined on the production of α-gal activity to induce the bacterial enzyme. Raffinose expressed the strongest induction, followed by starch, starch + raffinose, sucrose and sucrose + raffinose. Another time course experiment on the studied *Lactobacillus* revealed that consumption of raffinose increase the enzyme activity and gradual decrease in the activity was noted as the fermentation proceed. At the end, raffinose decreased to a non-detectable level in the medium (1.86 U/ml).

In a studied organism (*Lb.brevis*), α-galactosidase activity was assessed during fermentation including its growth and potential in utilizing oligosaccharide for development of bio-functional fermented pulse based product. *Lactobacillus* was successfully able to grow and utilize non-digestible oligosaccharide (NDO) from pulses. This confirmation was done by thin-layer chromatography and hydrolysis of raffinose and sucrose was observed in organism treated sample as against their level in untreated sample (control) indicating the reduction in NDO.

Thus, this would be advantageous in using single desirable organism in removing the oligosaccharide from such product and imparting various health benefits to the consumers.

Pulses are nutritionally rich in carbohydrates, proteins, minerals and vitamins. Productivity of microbial metabolites can be increased by manipulating nutritional requirements and physical parameters. Thus, development of economical medium requires selection of carbon and mineral elements. Lactic acid (LA) can be produced from abundantly available cheaper substrates like starch in a two-step process of saccharification followed by *Lactobacillus* fermentation. Direct conversion of carbohydrates to lactic acid by bacteria will eliminate the two-step process and make it more economical. Initial screening of the ingredients was done to understand the significance of their effect on the product formation and then a few better ingredients were selected for further optimization. In this study, a simplest synthetic medium consisting of pulses, yeast extract, and manganese sulphate as a carbon, nitrogen and mineral source, respectively, were used for bacterial growth, and high lactic acid production. Further, optimization was carried out with MRS components to produce the maximum amount of lactic acid. pH is an important factor for growth and LA production. During fermentation, pH profiles showed that medium with 20% PP maintained a relatively more acidic condition compared to other concentrations and growth of TIP1 was also inhibited. This may be due to unavailability of certain minerals in the medium, as *Lactobacillus* has a complex nutrient requirement, and the beans were dispensed only in distilled water devoid of nutrients. This explains the requirement for vitamins and certain specific amino acids for organism's growth, which may not be present in the beans. Several authors reported that the presence of peptides in YE, contribution of B- vitamin, purine and pyrimidine bases in the medium enhances growth of *Lactobacillus*. The slow cell growth during the pigeon pea fermentation may be due to the deficiency of nitrogen in the medium. Nancib et al., (2001) compared use of date juice and various other nitrogen sources with yeast extract in terms of their efficiency for higher acidity, but none of the used nitrogen sources showed acidity as high as that obtained with yeast extract (8). Mn^{2+} showed a positive effect on lactic acid production due to Mn^{2+} being a cofactor of several *Lactobacillus* enzymes such as the RNA polymerase, lactate dehydrogenase (LDH), NADH oxidase and superoxide dismutase. This allows improving the efficiency and productivity of the lactic fermentation. Cheng et al., (2014) demonstrate the effect of Mn^{2+} on stimulation of LDH by directing the conversion of pyruvic acid to lactic acid, and they show that the production of lactic acid decreases in the absence of

Mn2+ (9). Although, *Lb. plantarum* tolerated high concentrations of Mn^{2+}, its growth was affected. For that reason, it is necessary to adjust Mn2+ concentration to prevent a negative effect on lactic acid production. 6.30, 5.10, 2.34, 0.20 g/l at 24, 48, 72, 96 h for PP; 4.17, 3.52, 0.41, 0.34 g/l at 24, 48, 72, 96 h for RL; 3.06, 1.44, 0.21, 0.17 g/l at 24, 48, 72, 96 h for BG; 5.60, 2.14, 0.28, 0 g/l at 24, 48, 72, 96 h for BB.

Acetic acid was not detected at 24 h in any samples. However, accumulation of acetic acid and lactic acid depends mainly on the sugar metabolism and substrate supply of the starter culture. Literature reports that acetic acid is produced only in kimchi fermented by *Lb. mesenteroides* and not in *Lb. plantarum* fermentations, similar to the results of the present study. Another author reported the concentration of acids produced by lactobacilli species, is strain-dependent, but that strains studied have a lower concentration or no detectable level of acetic acid than lactic acid. Although low lactic acid can leads to increase concentration of by-products like ethanol, succinnic acid, butyric acid and propionic acid in hetero-fermentative strains. In fact in some case, acetic acid appeared after 48 h mostly via the citrate metabolism or the degradation of lactic acid produced which may justify the reduction of lactic acid concentration inoculated with *Lb. plantarum* and *Lb. brevis*. Literature suggests that medium pH had a significant impact on the final ethanol concentration in the medium. A reduction in the final ethanol produced was observed as the pH of the medium reduced. Lowering the medium pH reduces growth and metabolism of contaminating bacteria significantly, it also reduces the efficiency of bacteria to convert sugars to ethanol, which ultimately results in reduced ethanol yield. However, increasing carbohydrate content with optimum pH of 5.0 to 5.5 for ethanol production, the bacteria can be controlled efficiently and maximum ethanol production can be achieved.

For anti-nutritional evaluation of processed beans, it was concluded that roasting treatment resulted in significant effect on nutritional quality with effective anti-nutritional factors reduction. Hence the use of combined food processing technologies such as soaking and roasting can be used as a strategy to overcome problem of anti-nutritional factors in beans. Significant elimination of tannins was achieved by de-hulling implying that tannins were mainly in the seed coats. Dehulling was previously reported to substantially reduce the levels of tannins in beans. Fermentation resulted in a decrease in tannin content in all the fermentation batches. Higher tannin reduction in the batches occurred between 24 h and 36 h of fermentation. The

reduction of tannin as a result of fermentation can be attributed to the hydrolysis of poly-phenolic compounds of tannin complexes during fermentation.

5.32, 4.08, 3.70, 3.56 mg/100g in raw, dry roasting, soaked, dehulled and 2.47, 2.36, 2.30, 1.86, 1.22 for 12, 16, 18, 24, 36 h in PP; 11.66, 9.00, 8.42, 8.12 mg/100g in raw, dry roasting, soaked, dehulled and 6.76, 6.24, 6.18, 5.11, 3.60 for 12, 16, 18, 24, 36 h in RL; 9.30, 7.67, 6.42, 6.30 mg/100g in raw, dry roasting, soaked, dehulled and 4.40, 4.10, 3.82, 3.05, 1.79 for 12, 16, 18, 24, 36 h in BG; 18.26, 15.28, 14.44, 14.07 mg/100g in raw, dry roasting, soaked, dehulled and 12.62, 11.13, 10.89, 9.37, 6.02 for 12, 16, 18, 24, 36 h in BB tannin content in pulses.

59.20, 58.67, 55.41, 54.88 mg/100g in raw, dry roasting, soaked, dehulled and 50.55, 48.50, 48.21, 45.79, 42.60 for 12, 16, 18, 24, 36 h in PP; 142.18, 136.60, 133.22, 131.98 mg/100g in raw, dry roasting, soaked, dehulled and 123.30, 119.41, 118.60, 115.17, 111.86 for 12, 16, 18, 24, 36 h in RL; 42.67, 41.33, 38.80, 38.86 mg/100g in raw, dry roasting, soaked, dehulled and 30.70, 28.19, 27.22, 25.92, 23.32 for 12, 16, 18, 24, 36 h in BG; 94.00, 90.11, 88.63, 87.61/100g in raw, dry roasting, soaked, dehulled and 85.23, 83.22, 81.51, 79.57, 76.82 for 12, 16, 18, 24, 36 h in BB.

Conclusion

Lactobacillus is one of the most illustrative and common GRAS organism recognized in fermented food and thus it is important to address its effect in reducing NDO using different strains during preparation of pulse based product. In order to simply and effectively study the hydrolysis of oligosaccharide being work practically, a synthetic medium was designed with modified medium components. The reduction in oligosaccharides was observed as well as α-galactosidase enzyme was efficiently produced. However, when the same strain was applied to the food product, the enzyme α-galactosidase displayed higher activity compared to the previous study using modified medium. The result of this study showed the possibility of an organism to easily adopt in an environment and successfully able to reduce complex sugars into a simpler form. Further, optimization of medium for maximum lactic acid production was carried out; this study could produce lactic acid with higher concentration and productivity than the reported concentration making it more promising for future study and process improvement. Lastly, in anti-nutritional evaluation of processed beans, it was concluded that processing methods including fermentation resulted in significant effect on nutritional quality with effective anti-

nutritional factors reduction. Hence, it provides a good opportunity for a food based industry to develop a cost effective functional food rich in nutrition.

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