Probiotics are microorganisms that provide health benefits when ingested by humans and animals. The spectrum of probiotic activity can have nutritional, physiological and antimicrobial effects in humans and animals. Probiotics can also improve nutritional quality of food and feed, acts as metabolic stimuli of vitamin synthesis and enzyme production, stabilize gut microflora and competitively exclude enteric pathogens, enhance innate host defense by production of antimicrobial substances, reduce serum cholesterol by assimilation mechanisms, decrease risk of colon cancer by detoxification of carcinogens, tumor suppression by modulation of cell-mediated immunity. Probiotics are used in humans and animals for generations but they have recently been subjected to clinical research. Particularly, investigation of potential benefits of probiotics for the healthy consumer and market for probiotic products requires more attention and research. Also, the potential use of probiotics outside the gastrointestinal tract deserves further exploration. Most of the claims made by probiotic manufacturers are not supported by clinical data and require further studies. Well conducted clinical studies will result in increase of acceptance of probiotics for the treatment and prevention of selected diseases. The increased use of probiotics and prebiotics in food industry has lead to investigation of various biotechnological processes and search of new microorganisms. Study of enzymes of probiotic strains is also very interesting area as supply of enzymes is one of the mechanism of probiotic action. These enzymes help in imparting health benefits to host and some of them also improve the taste and texture of the food stuff in which probiotic strains are incorporated. Future challenges include the incorporation of one or more probiotics together or in combination with suitable prebiotic substrates to enhance the efficacy of the preparations for clinical use.

LAB is an important group of microorganisms as they are generally regarded as safe (GRAS) and play industrially important role in their use as probiotics. However, it is important to assess the safety of LAB to be used as food additive (probiotics). Mostly, potential of LAB to be used as probiotics is studied on the strains of human or animal origin as these strains are assumed to have adaptability of colonization in the human/animal GI tract. But few studies are engaged in determination of probiotic attributes of dairy originated strains under GI conditions. Probiotic action is species and
strain specific and single strain of probiotic can act via multiple, concomitant pathways. Probiotics have been used for generations as an alternative to traditional medicine for maintenance of enteric homeostasis and disease prevention. However, their mechanism of action and effectiveness of use is an area of interest. Investigation and development of a highly potent probiotics consortium suitable for functioning in adverse conditions can be of great value as therapeutics and can be comparable to commercially available products/Probiotics.

Presence of different enzyme activities is one of the mechanism of probiotic action. Enzymes of LAB are industrially important because they are from safe source. Present work deals with screening of LAB for the presence of PIP activity and purification, characterization of PIP from the selected strain and isolation, purification, characterization of β-galactosidase from P. acidilactici. Application studies of these two enzymes were carried out.

Different strains viz. L. acidophilus, L. rhamnosus, P. acidilactici, L. corniformis, L. fermentum, L. brevis, L. plantarum and L. paracasei were screened for the presence of PIP. Sonicated cells and their extracellular secretions were used for screening PIP activity. L. plantarum showed maximum PIP activity. Colorimetric method was used to assay PIP with synthetic substrates Pro-4-mβNA and the coloured product was observed at 520 nm. Membrane bound PIP from selected L. plantarum was further extracted and purified.

Since PIP of L. plantarum was membrane bound, its extraction and solubilization was a tough task. It was extracted by a previously developed protocol using sonication in the presence of salt and detergent and further freezing and thawing. The protocol is simple and straightforward eliminates the need of lengthy centrifugation/ultracentrifugation for the extraction of membrane enzyme(s).

Purification of extracted PIP was achieved in three chromatographic steps. Extracted enzyme was dialysed and subjected to CM-sephadex column chromatography at pH 5.9. Active fractions from this step were pooled, concentrated, dialysed and further subjected to Sephadex G-100 column chromatography at pH 7.4. Fractions containing the enzyme were again pooled concentrated, dialysed and loaded on Q-sepharose column chromatography at pH 7.5. Finally enzyme containing fractions were pooled and concentrated. PIP was purified by 7.13 purification fold with specific
activity of 26.4 U/mg and yield of 33.5%. PIP was checked for purity using Davis gel electrophoresis at pH 8.3 and single band confirmed its purity and homogeneity.

Molecular weight of the enzyme was determined and confirmed by SDS-PAGE and MALDI-TOF. The results confirmed that PIP is a homodimer with molecular weight of 37.9 kDa and subunit mass of 18.9 kDa. The pH optima of PIP (with Pro-4-\text{mβNA}) was 7.0. PIP showed 90% and more activity in a pH range of 5.5-8.0. Optimum temperature studies revealed that PIP was maximally active at 45°C. Since the substrate Pro-4-\text{mβNA} was dissolved in DMSO, the effect of DMSO was studied on enzyme activity. Activity of PIP initially increased with 1% DMSO concentration but further increase in DMSO concentration was accompanied with decreased enzyme activity. Only 60% activity was retained at 4% DMSO concentration. At low concentration increased substrate solubility might cause increase in activity. Disturbance of conformation of hydrophobic residues at high DMSO concentration might be a cause of decreased enzyme activity. A wide range of synthetic substrates were analyzed for substrate specificity of PIP Amongst the various substrates, PIP showed remarkable activity only against Pro-4-\text{mβNA}. The enzyme exhibited narrow substrate specificity with preference for Pro-4-\text{mβNA} with little or no hydrolysis of other studied substrates. The kinetic parameters \( K_m \) and \( V_{\text{max}} \) of PIP was found to be 65 μM (\( K_m \)) and 25.9 nmoles/min/ml (\( V_{\text{max}} \)) respectively. These values were determined using Line Weaver Burk plot, Hanes plot and Michaelis-Menten plot. To classify the enzymes on the basis of catalysis their inhibition was studied with different chemical inhibitors. Strong inhibition of PIP by PMSF confirms that the enzyme was a serine protease and inhibition by DEPC suggests involvement of His in enzyme catalysis in both cases. Purified PIP was also strongly inhibited by reducing agents including cysteine, glutathione and DTT which indicated the involvement of sulfhydryl groups in either catalytic mechanism or enzyme regulation. None of studied metal ions affected the activity of PIP. This suggests that PIP is neither a metalloprotease nor metal dependent. \textit{In-silico} evaluation of the enzyme was also performed. 3D modelling and phylogenetic analysis of the enzyme was also carried out.

Enzymatic hydrolysis of collagen and/or gelatin results in production of bioactive peptides and free amino acids. Hydrolyzed collagen have high amounts of glycine, lysine and proline/hydroxyproline, that are found in lower amounts in other
proteinaceous food supplements. Collagen hydrolysates are easily digested because of their low molecular weight and are quickly absorbed. These products replace the synovial fluids between the joints and repair and build cartilages weakened by overuse through impact and stress. They benefit hair, skin tissue, muscle, cartilage, ligaments and blood cell growth and also have anti-aging properties. Collagen is rich in proline and hydroxyproline and likely to be hydrolysed by PIP. Hydrolytic activity of PIP for collagen was studied by combining with other enzymes. Neutral protease generates oligopeptides which are further cleaved by exopeptidases. DPP-II is an exopeptidase that removes dipeptide moieties from oligopeptides and also removes proline and hydroxyproline at penultimate position. So enzyme cocktail of DPP-II, purified from *Pediococcus acidilactici*, neutral endoprotease and PIP (from *L. Plantarum*) was used to hydrolyse collagen. Free amino acid content was analysed using ninhydrin test, TLC and HPLC. Ninhydrin test confirmed that when PIP was used in combination with neutral protease and with neutral protease and DPP-II the amount of free amino acids increased as compared to PIP and neutral protease alone. The release of free amino acids was maximum when all enzymes were used in combination. HPLC results revealed the release of good amount of essential amino acids lysine (20.25 µg/ml), valine (34.89 µg/ml) and histidine (6.4 µg/ml) when PIP was used in combination with neutral protease. Isoleucine (67.69 µg/ml), phenylalanine (52.955 µg/ml) were released when all enzymes were used and leucine (112.294 µg/ml) released in good amounts when PIP was used alone. Ornithine (26.321 µg/ml), which has antifatigue effect and helpful in increasing level of human growth hormone, act as a precursor of polyamines. OH-Lysine (71.65 µg/ml) contents increased in case of treatment with PIP and neutral protease. The use of combination of all enzymes resulted in release of aspartic acid (4.015 µg/ml) and anserine (4.472 µg/ml), which act as an antioxidant. OH-Proline (1.241 µg/ml) is released when collagen was treated with PIP and neutral protease. There are also reports focused on the studies of types of bioactivities of collagen hydrolysates viz. antimicrobial, antioxidant or antihypertensive activities and on the effect of oral intake of hydroxyl proline containing peptides. Waste waters of butcheries and meat industries are rich in collagen, therefore hydrolysis of collagen can also help in wastewater treatment and for production of bioactive peptides and free amino acids.
Use of proteolytic enzymes is popular method for meat tenderization, therefore, applicability of purified PIP was also studied with respect to meat tenderization, in order to improve meat quality and palatability. Meat tenderization was studied after treatment with purified PIP alone and after treatment of meat with PIP in combination with other enzymes. Whole sonicated cells of *L. plantarum* were also used for the purpose. Results of SDS-PAGE and microscopic analysis revealed that PIP helps in meat tenderization, in purified state as well as in membrane bound form of sonicated *L. plantarum* cells. The effectiveness of sonicated cells of *L. plantarum* (a probiotic LAB) in collagen degradation and meat tenderization marks its industrial importance. This observation can be very useful for food and meat industry in addition to waste treatment of meat industry. There are also reports of debittering of fermented dairy products by proline specific enzymes. Diet supplements of collagen hydrolysates resulted in reduction of pain in patients suffering from osteoarthritis, osteoporosis and in physically active athletes with activity related joint pain.

β-galactosidase from *P. acidilactici* was extracted, purified and characterized. Colorimetric method was used to assay β-galactosidase using ONPG substrate and the coloured product was read at 420 nm. For extraction of intracellular β-galactosidase of *P. acidilactici* several methods including sonication, lysozyme-EDTA treatment, SDS-chloroform treatment and lysozyme treatment were used because method of extraction of this enzyme vary from strain to strain. β-galactosidase was maximally extracted via sonication method. Extracted β-galactosidase was subjected to 0-80% (NH₄)₂SO₄ fractionation and then concentrated and dialysed enzyme was loaded on Sephadex G-100 column for gel filtration chromatography at pH 7.0. Fractions containing the enzyme were pooled concentrated and dialyzed at pH 6.5. The concentrated enzyme from the previous step was loaded on Q-sepharose column for anion exchange chromatography at pH 6.5. The active fractions were then pooled and concentrated. β-galactosidase from *P. acidilactici* was purified with 3.06 fold purification with specific activity of 0.883 U/mg and yield of 28.26 %. A single band on native-PAGE confirmed the purity of the enzyme. The pH optima of β-galactosidase was 6.0. β-galactosidase retained 75% activity at pH 5.4 and upto 50 % activity at pH 8.0 while optimum temperature studies revealed that β-galactosidase was maximally active at 50°C. Higher temperature optima and pH stability range mark industrial importance of β-
galactosidase. $K_m$ and $V_{\text{max}}$ of $\beta$-galactosidase were found to be 400 $\mu$M ($K_m$) and $1.221 \times 10^{-1}$ U ($V_{\text{max}}$). Strong inhibition of $\beta$-galactosidase by PMSF confirms the enzymes as a serine protease and inhibition by DEPC suggests involvement of His in enzyme catalysis. Activity of $\beta$-galactosidase was only slightly increased by presence of $\text{Mn}^{2+}$ and $\text{Mg}^{2+}$. In-silico evaluation involving 3D modelling and phylogenetic analysis of the enzyme was also done.

The ability of purified $\beta$-galactosidase for lactose hydrolysis in milk was studied using defatted milk as substrate. Rate of lactose hydrolysis and $t_{1/2}$ were found to be 0.047 min$^{-1}$ and 14.74 min respectively. Lactose hydrolysis increased up to 65 min of reaction time and about 98 % lactose had been converted. The enzyme effectively degraded milk lactose with good rate of hydrolysis and $t_{1/2}$.

$\beta$-galactosidase can be used in transglycosylation of lactose to synthesize galacto-oligosaccharides (GOSs). These are prebiotics not hydrolyzed or absorbed in the upper intestinal tract, and they pass onto the colon where they are fermented selectively by beneficial intestinal bacteria. Besides their prebiotic effects, these GOSs have anticarcinogenic properties and low caloric values. Pre- and probiotics both result in beneficial effects to the host after consumption. Combinations of pre- and probiotics confer synergistic effects and are called as synbiotics. Therefore, synthesis of GOSs was studied using $\beta$-galactosidase enzyme under study. Lactose was used as substrate for the purpose and response surface methodology was applied to optimize the production of GOSs. The results were analysed by TLC. The production was studied upto 24 h and 90% of products were formed within 4 h. 30% lactose and 2.75 units of enzymes were suitable for GOS production.

In-vivo studies are needed to establish $P. \text{acidilactici}$ as a probiotic strain. However, preliminary studies established it to be a potential probiotic and opened avenues for development of new probiotic foods. The possibility of formation of mono-strains into multi-strain probiotics with the potential of improved efficacy is the goal of this kind of studies. $\beta$-galactosidase enzyme is industrially very important. It can be used to avoid crystallization of lactose in concentrated frozen dairy products such as condensed milk, ice creams etc., it helps in assimilation of lactose containing food in lactose-intolerant population, in solving problem of whey disposal by converting whey into different value added products, in production of biologically active galacto-
oligosaccharides that can contribute to human health when used as prebiotic functional food ingredients.

Whey is the major byproduct of dairy industry and retains 55% of milk’s nutrients including lactose, soluble proteins, lipids and minerals. It also poses the major disposal problems because of its volume and high biochemical oxygen demand. The problem of whey disposal can be solved by converting whey into different value added products and lactic acid is one of them. Lactic acid (2-hydroxy propanoic acid) and its derivatives have gained prominence because of their applications in food, pharmaceutical, textile, cosmetic and chemical industries. Presence of milk’s nutrients in whey makes whey, a suitable substrate for fermentation by LAB. Hence lactic acid production from whey was studied and optimized using LAB viz. *Pediococcus acidilactici* and *Lactobacillus plantarum*. Lactic acid production from sugarcane juice and biowastes including sugarcane bagasse, apple pomace and grape pomace was also studied under optimized conditions. Biowastes were used without pretreatment. All the studied substrates efficiently produced lactic acid and apple pomace resulted in maximum yield (>30 g/l). Therefore, fermentation of biological wastes serves dual purpose of lactic acid production and waste disposal by using LAB which are generally regarded as safe (GRAS).

Effective enzyme systems and enzyme activities is one of a mechanism of action of probiotic bacteria. The studies of these enzymes will help in their utilization for different industrial purpose. Further work is needed to explore other probiotic activities of *P. acidilactici* that can further promote it in process of being establishing as a probiotic strain. Study of activities of other probiotic strains in combination with *P. acidilactici* can also help in production of a combination of probiotics with maximum health benefits. Future studies of incorporation of one or more probiotics together with enhanced beneficial health effects because of different enzyme activities, can open doors for the production of very promising probiotic fermented foods.