Production of functional food, Fruit ravioli with antihypertensive peptides

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CERTIFICATE

This is to certify that the thesis entitled "**PRODUCTION OF FUNCTIONAL FOOD, FRUIT RAVIOLI WITH ANTIHYPERTENSIVE PEPTIDES**" which is being submitted by **Ms. Preeti Acharya**, Roll No. **412LS2031**, for the degree of Masters of Science in Life Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

R. Jayabalan 11/05/2014.

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DECLARATION

I hereby declare that the thesis entitled **"Production of functional food, Fruit ravioli with antihypertensive peptides"**, that I submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a record of bonafied and original research work carried out by me under the guidance and supervision of Dr. R. Jayabalan, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge no part of this thesis has been submitted to any other university or institution for the award of any degree or diploma.

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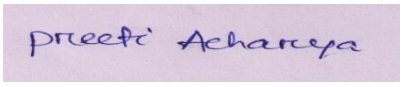

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ABSRACT

Hypertension is one of the major independent risk factor for cardiovascular diseases. Angiotensin converting enzyme (ACE) plays an important role in regulation of blood pressure as well as cardiovascular function. It converts angiotensin I into angiotensin II in blood, which causes the muscles surrounding blood vessels to contract and narrow the vessels thus increasing the pressure within the blood vessels and may lead to hypertension. ACE was also reported to inactivate bradykinin, a vasodilator. ACE inhibitors have achieved widespread usage in the treatment of cardiovascular and renal diseases mainly in the treatment of hypertension. In the present study, bacteria isolated from OMFED curd were utilized to produce anti-hypertensive peptides from milk. The peptides were used for the development of a novel physiologically functional food, watermelon ravioli, having anti-hypertensive property. ACE cleaves hippuryl -L-histidyl-L-leucine (HHL) into hippuric acid (HA) and histidyl leucine (HL). Production of anti-hypertensive peptides was evaluated by direct spectrophotometric determination of HA. The extent of HA released is directly propotional to ACE activity. The amount of HA was determined by using HA standard graph. Inhibitory activity of the anti-hypertensive peptides produced by fermentation of milk in presencce of the isolated bacteria, was compared to a known inhibitor of ACE (captopril).

Key words: Angiotensin converting enzyme (ACE), ACE inhibitor, Hippuryl-L-histidyl-L-leucine, hippuric acid, antihypertensive peptide.

INTRODUCTION

Probiotics are living organisms defined as a viable microbial dietary supplement which has a beneficial effect on the host through intestinal tract. It has also been proposed to be "a live microbial food ingredient that is beneficial to health" [Salminen et. al., 1998]. These are both gram positive & gram negative which are used in constipation & colities, play an important role in immunological modulation, respiratory , gastrointestinal function , alleviation of diarrhoea, improvement of irritable bowel, lactose intolerance, antimicrobial properties , etc. [Floch et. al., 2011] . It gives protection by competing with intestinal pathogens through the release of bacteriocin, an antibacterial substance [Cotter et. al., 2005], also some metabolites like lactic acid and acetic acid [Servin, 2004]. The main action of probiotic is reinforcement of the intestinal mucosal barrier against various agents. It reduce pathological alteration in paracellular permeability to large molecules & also mucosal immunity is stimulated, reduce mucus degradation & interact with mediators of inflammation [Jean et. al., 2003]. The most common probiotic bacteria are *Lactobacillus* and *Bifidobacterium* used for the production of food like yogurt, cheese, pickle etc. However, recent studies have suggested that probiotics could have beneficial effects beyond gastrointestinal health, as they were found to improve certain metabolic disorders such as hypertension. Hypertension is caused by various factors and the predominant causes include an increase in cholesterol levels, incidence of diabetes, inconsistent modulation of renin and imbalanced sexual hormones. Cardiovascular diseases like coronary heart disease, atherosclerosis, stroke, and heart failure are a major health concern, because they are one of the leading causes of death. The major risk factor of cardiovascular diseases is hypertension [Chrysant et al., 2013] which is estimated to be affecting about 20% of world's population (Jordan et al., 2001). Hypertension is defined by the World Health Organisation (WHO) as the exceeding of 90 mmHg for the diastolic arterial pressure, and 140 mmHg for the systolic pressure. Angiotensin I-converting enzyme (ACE, dipeptidyl carboxypeptidase, EC 3.4.15.1) plays an important role in the regulation of blood pressure as well as cardiovascular function. ACE converts the inactive decapeptide angiotensin I into the potent vaso-constricting octapeptide angiotensin II and also inactivates vasodilator, bradykinin (Na et al., 2000). Inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension. Therefore, in the development of drugs to control high blood pressure, ACE inhibition has become an important activity. Many studies have been attempted in the synthesis of ACE inhibitors such as captopril, enalapril,

alcacepril and lisinopril, which are currently used in the treatment of essential hypertension and heart failure in humans (Schmidt et al 2014,). However, these synthetic drugs are believed to have certain side effects such as cough, taste disturbances, skin rashes or angioneurotic edema all of which might be intrinsically linked to synthetic ACE inhibitors (Atkinson and Robertson, 1979). Therefore, the research and development to find safer, innovative, and economical ACE inhibitors is necessary for the prevention and remedy of hypertension [Anne et al., 2011]. Many research groups have combed for novel ACE inhibitors from natural products [Gasse et al., 1984], microbial sources and food proteins. Functional foods, beneficial to health, are having a marked effect on the food sector. The production of functional foods has paid special attention to the study of the physiological role played by dietary proteins. There are certain fragments within the sequence of food proteins that may show biological activity once released by hydrolysis. These fragments, known as bioactive peptides, can be produced in vivo by the action of gastrointestinal enzymes and can also be obtained in vitro using specific enzymes, or during the fermentation of certain foods. In the present study we are focusing on development of watermelon raviolis, a functional food consisting of anti-hypertensive peptides by the process of encapsulation. Here we developed the beads of milk fermented with the proteolytic isolates which were again encapsulated along with fruit juice (watermelon) and honey. As we know the survival of bacteria inside the upper gastrointestinal tract is poor due to the acidic and enzymatic secretions, hence in order to provide protection against several environmental factors inside GIT encapsulation method is adopted. By creating a microenvironment where the bacteria will survive during processing and storage while being released to particular site in the intestine.

REVIEW OF LITERATURE

Probiotics are usually defined as living microbial dietary adjuvants, which has a beneficial effect on human physiology by improving nutritional and microbial balance in the intestinal tract [Fuller, 1989; Guarner and Schaafsma, 1998]. After birth these microbial flora inhabitate the human intestinal tract. About 400 species of various commensal microorganisms whether bacteria, viruses, yeast lived in the digestive tract [Moore and Holdeman, 1974; Koop-Hoolihan, 2001]. The grandfather of modern probiotic Eli Metchnikoff discovered that the lactic acid bacteria (LAB) can have a positive influence on digestion and the immune system [Anukam and Reid, 2008]. Probiotic microorganisms may be gram-positive (*Lactobacillus*) or gram- negative (*Escheria coli*) and are used in the treatment of intestinal dysfunction [Marco, Pavan and Kleerebezem, 2006] and treatment of chronic constipation [Mollenbrink and Bruckschen, 1994] and colitis. Probiotics are beneficial components that have been used to modulate gastrointestinal health like improving lactose intolerance, increasing infection resistance, suppression of pathogenic bacteria etc. Currently the most important applications of probiotics involve benefits like anti-hypertension, immuno-modulation, improving serum lipid profiles & alleviation of post menopausal disorder.

For the evaluation of health and nutritional properties of probiotics, first of all these should not posses pathogenic character, not capable of transferring antibiotic resistance to pathogen, then it also should not be the source of antibiotic resistance [Joint FAO/WHO Expert consultation on evaluation of health and nutritional properties of probiotics in Food including power milk with live lactic acid bacteria. Probiotic like Lactic acid bacteria produces a range of short chain fatty acid like acetic, lactic, butyric and propionic acid during fermentation, which reduces the intestinal milieu pH and has inhibitory effect against pathogenic bacteria. Normally Lactic acid & acetic acid inhibit the growth of pathogen like *Staphylococcus aureus, Salmonella typhimurium, Campylobacter shigella* etc. Hydrogen peroxide, produced by Lactic acid bacteria, controls the growth of *Candida albicans*.

About 70% of world's population have low amount of beta-galactosidase leading to lactose intolerance where lactose behaves like osmotic, non-digestible carbohydrate. Probiotics are used to improve lactose digestion by producing beta-galactosidase enzyme thereby reducing the intolerance & also slowing oro-cecal transit [Sanders, 1993]. These

bacteria play an important role in functioning of mucosal immune system. In case of infants for the enhancement of immunoglobulin-A, *Lactobacillus casei* strain are supplemented. The major habitat for probiotic bacteria is intestinal mucosa, so they closely associate with gut associated lymphoid tissue (GALT), because it constitutes largest lymphoid tissue in human body.

Another application of Probiotic bacteria is the reduction of serum lipid level. By several mechanisms LAB can reduce the cholesterol and lipid level such as direct assimilation of cholesterol, deconjugation of bile salts and reduced transport of cholesterol to plaque deposits and inhibit the formation of low density lipoprotein [Fuller R., 1989]. When beta-hydroxyl-beta-methyl-glutaril- coenzyme A reductase is consumed with probiotic in liver, it will decrease the cholesterol synthesis. Probiotic help in balancing the intestinal flora thus reduce the risk of diarrhoea, stress, antibiotic effects, *Candida* infection and food allergy. These strains are also used for urogenital therapy by attaching them to vaginal and uro-epithelial cells as they are able to interfere with adhesion of pathogens.

Most of fermented dairy products or dietary supplements, such as yogurt and kefir contain probiotic organisms [Salminen, Isolauri and Salminen, 1996] such as *Lactobacillus, Bifidobacterium, Streptococcus thermophiles and Bacillus spp.* Yogurt, the primary food containing probiotics, is produced by fermentation of milk using 2 starter cultures, *Lactobacillus bulgaricus & Streptococcus thermophilus* .

Milk, an excellent source of proteins and peptides, exhibit a range of biological activities influencing digestion. The specific functions of milk include supply of amino acids, nitrogen and proteins which provide protection, micelle formation and facilitate the uptake of several necessary nutrients such as trace elements and vitamins. Milk proteins are highly functional due to presence of biologically active peptides having anti-hypertensive, cyto-modulatory, hypo-cholesterolemic, anti-oxidative, anti-thrombotic, immuno-stimulating, anti-microbial, mineral carrying and cholesterol lowering activity [Shah, 2000]. By proteolytic hydrolysis or enzymatic digestion, many bioactive peptides are produced from milk [Kamau et.al, 2010]. The type & density of peptides released depend upon the quantity and quality of milk, presence of additional food, pH and enzymatic action. The Fermented milk products have high nutritional value, which will give rise to many health-promoting effects, like improvement of lactose metabolism, serum cholesterol level reduction and decrease the risk of cancer [Shah, 2007]. LAB can hydrolyze casein protein into peptides and amino acids during normal fermentation process. Bioactive peptides are produced by both starter and non-starter bacteria like *Lb. helveticus, Lb. delbrueckii* ssp *bulgaricus, L. lactis*

ssp. *diacetylactis, L. lactis* ssp. *cremoris* and *Streptococcus (Str.) salivarius* ssp. *thermophilus* which are used to hydrolyze milk proteins and release ACEI (Angiotensin converting enzyme inhibitor) peptides. The bacterial cell consists of cell wall-bound proteinase and a number of distinct intracellular peptidases, endopeptidases, aminopeptidases, tripeptidases and dipeptidases [Christensen et.al, 1999]. One of the bacterial strains of *Lb. helveticus* produces oligo-peptides by the digestion of milk proteins [Foucaud and Juillard, 2000] which is known to have high proteolytic activities [Luoma et.al, 2001]. The activities of peptidases depend upon the growth conditions which makes it possible for the formation of peptides [Williams et.al, 2002].

Bioactive peptides, specific protein fragments having positive impact on body functions and conditions, may ultimately influence health [Fitzgerald and Murray, 2006]. Bioactive peptides may affect major body systems like cardiovascular, digestive, immune and nervous system. These beneficial health effects are anti-microbial, anti-oxidative, anti-hypertensive and immuno-modulatory [Fitz and Meisei, 2003].

The liberated bioactive peptides may act as regulatory compounds like hormones. There are numerous peptides isolated, which exhibit various activities such as opiate, anti-thrombotic or anti-hypertensive activity, immune-modulation or mineral utilization properties etc. Though other animal as well as plant proteins also contain bioactive peptide sequences but milk proteins are the important source for the production of bioactive peptides. The first biologically active peptides found in milk were opioid peptides and immuno-modulatory peptides. Opioid peptides in dairy products have an important role in nervous system [Teschemacher, 1997]. They are short sequences of amino acids which mimic the effect of opiate in the brain. Brain opioid peptide system play specific role in motivation, emotion, stress, pain and also control the food intake [Nakamura et.al, 1995]. The potency of bioactivity of peptides is inversely related to chain length. Angiotensin II can affect bone by decreasing the osteoplast differentiation and increasing osteoclastic bone resorption. So for the formation of bone, ACE inhibition is required which increases the vascular endothelial growth factor formation [Hiruma et al., 1998; Hatton et al., 1997]. ACE inhibition can also multiply the activity of bradykinin which has a specific role in bone formation. Immuno-modulatory peptides help in proliferation of lymphocytes, phagocytic activities of macrophages and antibody synthesis. These peptides also help in proliferation and maturation of T-cells and natural killer cells for defence mechanism against enteric bacteria [Clare and Swaisgood, 2000]. Also immune-modulatory milk peptides alleviate the allergic reaction and

increase the mucosal immunity in digestive tract which regulates the growth of immune system in infants.

There are two well-known peptides isolated from sour milk which have ACE inhibitory activity, val-pro-pro (VPP) and ile-pro-pro (IPP) derived from casein. Whey protein derived peptides may also show ACE inhibition. The ACE inhibitory peptides have C-terminal amino acid sequence which is hydrophobic and can compete with substrate as the side chains of these amino acids interact with the active site of ACE. For ACE inhibition C-terminal tryptophan, proline, tyrosine and phenylalanine are the most effective amino acid residues. Immuno-modulating peptides are detected in human and cow milk proteins [Migliore-Samour and Jolls, 1988]. A group of peptides shows the inhibition of ACE in favour of formation of bradykinin, acting as immuno-modulator [Pagelow and Werner, 1986]. Casein derived peptides have very interesting applications in dietary supplements and pharmaceutical preparations such as tablets, toothpaste etc.

Antihypertensive peptides:

Hypertension is a multifactor disorder that is caused by an interaction between environmental factors and genetic background. As there are various type of anti-hypertensive drugs discovered [Arsenault et.al, 2010], lacto-tripeptides have an inhibitory activity against ACE. Several studies have suggested that ACE activity is not affected by the oral tri-peptide administration so other mechanisms are needed to be applied for the reduction of blood pressure. As we know cardiovascular disease (CVD) is one of the leading and targeting diseases in world causing death of both males and females. So far, for the reduction of CVD risk on human health, improved diet and lifestyle has been adopted. The most independent risk factor for CVD is hypertension and Angiotensin I-converting enzyme (ACE) plays an important role in blood pressure controlling by converting angiotensin I into angiotensin II (a vasoconstrictor) and it also inactivates bradykinin (a vasodilator) [Harris et al.,1985]. Various synthetic ACE inhibitors like captopril, enalapril, lisinopril, and ramipril have been widely used in the clinical treatment of hypertension and heart failure in humans. But these synthetic ACE inhibitors can have side effects including cough, taste disturbances and skin rashes [Messerli and Hypertens, 1999]. So the natural ACE inhibitors are inevitable to rule out further complications. The antihypertensive peptides in fermented food products are reported to be very effective natural ACE inhibitors. Many ACE inhibitory peptides have been reported to be formed by enzymatic hydrolysis of natural food protein like casein, whey protein fish protein soy bean protein and corn gluten. The primary source of antihypertensive

peptides is dairy products like cheese and yogurt. Sour milk fermented with *Lb. helveticus* contains lacto-tripeptides, IPP and VPP, well known ACE inhibitors. The amount of IPP and VPP increases during the cheese ripening process. Whey protein produces an anti-hypertensive dipeptide Tyr-pro. From the parent protein peptides are delivered either by enzymatic hydrolysis during gastrointestinal digestion, fermentation with proteolytic starter cultures or hydrolysis by enzymes obtained from micro-organisms. If the structure of these peptides is known, it is also possible to synthesize peptides by chemical synthesis, recombinant DNA technology or enzymatic synthesis. When the isoelectric casein is hydrolysed with pepsin it generates anti-hypertensive peptides, some of the potent ACE-inhibitory peptides other than the two tripeptides are RYLGY, AYFYPEL and YQKFPQY, which have in vitro radical scavenging activity and anti-hypertensive activity. These peptide sequences are resistant to gastrointestinal enzymatic activity. Thus in our body blood pressure is partially controlled by renin angiotensin system (RAS).

ACE is a multifunctional ecto-enzyme. It is a bivalent dipeptidyl carboxyl metallo-peptidase which is present in the membrane-bound form in endothelial, epithelial or neuro-epithelial cells [Skidgel and Erdos, 1993], located in different tissues. The increase and decrease in blood pressure conditions is especially dependent on ACE activity. The greater the ACE activity, the more angiotensin I is converted to angiotensin II, which induces high blood pressure. ACE converts angiotensin I into angiotensin II by removing the C-terminal dipeptide HL, rennin act on angiotensinogen which is an inactive precursor. ACE also breaks down the bradykinin which is a vasodilator. Smooth muscle has AT1 receptors coupled with Gq protein, bind to arteries and veins which are constricted by Angiotensin II. Angiotensin II facilitates the release of norepinephrine from adrenergic nerves and inhibits the reuptake of norepinephrine.Fig-1 shows the mechanism of ACE activity.

Hence inhibition of ACE results in lowering hypertension. ACE inhibitor are help in dilating arteries and veins by inhibiting the metabolism of bradykinin. Vasodilation reduces the arterial blood pressure. It also down regulates the sympathetic adrenergic activity with the blockage of angiotensin II .

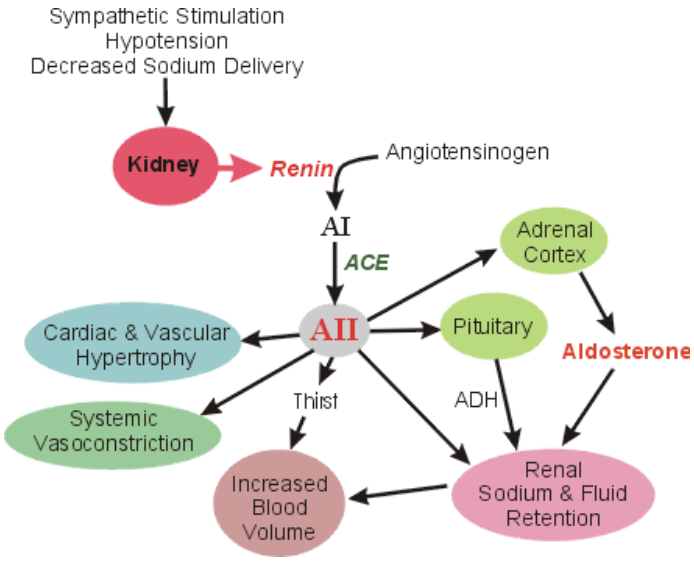


Fig-1: Mechanism of ACE activity

ACEI have natriuretic and diuretic effects, can also promote renal excretion of sodium and water by blocking the effects of angiotensin II on kidney and aldosterone secretion. Therefore ACE inhibition gives rise to hypotensive effect that influences the immune-defence and nervous system activity. These peptides are showing the antihypertensive effect after 2-8 hour of administration. Studies show that the anti-hypertensive effect of dose dependent peptide in spontaneous hypertensive rats was from 0.2-2 mg/kg of body weight dosage level when administered orally. Calpis sour milk contains IPP and VPP tripeptides which is administrated can decrease the systolic blood pressure within 2-4 weeks. ACE inhibitory peptides from milk cannot be used as drug for the hypertension treatment but it can be used as natural therapeutic agent without side effects. Normally the effect of ACE inhibitory peptides from the dairy product is totally depend upon the ability to reach the target site without any degradation or any inactivation by the GIT enzyme, peptidases. So ACEI peptides should be resistant to peptidase degradation such as the tripeptides contain C-terminal prolines are resistant to tryptic peptidases. Beta-lactoglobulin fragment f142-f148 is resistant to pepsin and chymotrypsin degradation, which confirms that its ACE inhibitory activity will be more. To exert an antihypertensive effect, ACE inhibitory peptides should reach the cardiovascular region in an active form. So these peptides should not be damaged by human proteases and transported actively through the intestinal wall to the blood. Therefore, it is necessary to study the bioavailability of ACEI peptides.

There are several methods developed to measure ACE inhibitory activity, like spectrophotometric fluorometric radiochemical, capillary electrophoresis and high-performance liquid chromatography (HPLC) But HPLC and capillary electrophoresis, radiochemical method requires expensive instruments which are not available in all laboratories. The most accepted method described by described by Cushman and Cheung is spectrophotometric method used for the determination of ACE activity and inhibition in vitro. It is based on the hydrolysis of hippuryl-l-histidyl-l-leucine (HHL) by ACE to hippuric acid (HA) and histidyl-leucine (HL) as by products. The amount of HA released from HHL is directly proportional to the ACE activity. This spectrophotometric method, however, involves several tedious steps, these are to extract HA with ethyl acetate, then evaporation, re-dissolution in water, measure the absorbance of HA at 228 nm, and moreover, ethyl acetate is able to extract un-hydrolyzed HHL, which also absorbs strongly at 228 nm, thus overestimating the ACE activity.

In this method, the HA concentration from HHL was detected by the action of ACE in the presence of ACE inhibitors and antibody synthesis. The peptides may stimulate the proliferation and maturation of T cells and natural killer cells for defence of new born against a large number of bacteria, particularly enteric bacteria. Also, it has been suggested that immune-modulatory milk peptides may alleviate allergic reactions in atopic humans and enhance mucosal immunity in the gastrointestinal tract. In this way, immune-modulatory peptides may regulate the development of the immune system in newborn infants. Furthermore, immune-peptides formed during milk fermentation have been shown to contribute to the antitumor effects [Matar et al., 2003].

Probiotics are used in production of functional foods, pharmaceutical and therapeutic products. The beneficial effects of probiotic micro-organisms appear when they arrive in the intestine. The probiotics play important role in digestion and development of the gut microflora by modification of the composition and metabolic activity of the endogenous intestinal microbiota and preventing the overgrowth and colonization of pathogens by stimulating the immune system producing anti-bacterial substances (such as bacteriocins and hydrogen peroxide), acids (that reduce the pH of the intestine) and competing for nutrients. It has been suggested in several studies that some specific changes in gut microbiota composition are associated with different diseases [Finegold et al., 2010]. In order to produce health effective functional foods, these probiotics should be stored in the viable form until consumption and their viability should be maintained throughout GIT since several reports suggest that probiotics are poor in surviving environmental changes. So to provide a

stable environment, these probiotics are provided with a physical barrier to resist harsh environmental condition in GIT, adopting a specific method called as encapsulation.

It is the process involving entrapment of active organisms within a carrier and is used to improve living cell count in the functional food, extend their storage life and give them a significant form. In other words, it is the method of encapsulating liquid substance within solid shell. The goal of this process is to create a micro-environment for the survival of bacteria during processing and storage as well as passage through the upper part of the GIT. The major application is to protect against low gastric pH and digestive enzymes. The outer covering shell is made up of various polymers, carbohydrates, fats and waxes mainly dependent on the core material to be protected. Encapsulation technologies can be used in many applications in food industry like controlling oxidative reaction, maintaining colours, odours, flavours, providing sustained and controlled release and extending shelf life. The shell of sealed capsules should be semi-permeable, thin but strong, but it is designed in such a way that it releases the probiotic cells in the large intestine. In order to protect bioactive compounds such as vitamins, antioxidants, proteins and lipids, several encapsulation technologies are used for the production of functional foods with enhanced functionality and stability. The best encapsulation technology should be selected for probiotics in order to guarantee the survival of bacteria during the encapsulation process, in storage conditions and in gut after consumption.

Ionic hydrogel method using sodium alginate is the biopolymer mostly used for encapsulation. These are marine polysaccharides naturally occurring as capsular polysaccharide in bacteria and these polymer is made up of alginic acid, consisting of a linear binary copolymer of α-L-glucuronic acid and C-5 epimer of ß-D-mannuronic acid which are bound by 1-4 glycosidic bonds. Probiotic cell is immobilised in alginate due to its rapid, versatile and nontoxic nature. When alginate solution dissolves in water, the viscosity as well as the length of macromolecules increases. Alginates precipitate by crosslinking, gelling with divalent ions like Ca^{2+}, Ba^{2+} etc. The gelling occurs when the Ca^{2+} ions bind in alginate giving rise to three dimensional structures. Alginate can easily form gel matrix around bacterial cell, it is edible, safe for consumption, cheap, mild temperature needed for their function, properly dissolve in intestine and release the entrapped cells.

Generally milk proteins have excellent biocompatibility and are natural vehicles for probiotic cells, so they can be used as a delivery system.

OBJECTIVES OF THE PROJECT

The main objectives are

- To Isolate Lactic acid bacteria (LAB) from Omfed curd, Rourkela.
- To test for proteolytic activity of the isolates.
- To determine ACE inhibitory activity by direct spectrophotometric method.
- To develop watermelon raviolis, a functional food conferring anti-hypertensive effects of bioactive peptides formed by fermented milk as well as nutritional values of the fruit.

MATERIALS AND METHODS

3.1. Isolation of lactic acid bacteria from OMFED curd

OMFED curd sample was collected and serially diluted up to 10^{-7}dilution in each tube contained 9 ml of distilled water. 0.1 ml of diluted samples was spread over the surface of MRS agar plates (Table 1) and incubated at 37ºC for 48 hours. After incubation, pure cultures were prepared and stored in MRS agar slants. Morphology of the isolates was studied by gram-staining method.

Table-1: Composition of MRS agar media

Constituents	Gram/litre
proteose peptone	10
Beef extract	10
Yeast extract	5
Dextrose	20
Polysorbate 80	1
Ammonium citrate	2
Sodium acetate	5
Magnesium sulphate	0.1
Manganese sulphate	0.05
Dipotassium phosphate	2
Agar	12
Final p^H (at 25ºC)	6.5

3.2. Screening for proteolytic activity

Isolates were streaked on gelatin nutrient agar media (Table 2). Plates were incubated at 30ºC for 48 hours. After incubation the plates were flooded with 5 ml mercuric chloride solution (15 g of $HgCl_2$ and 20 ml of 6 N HCl in 100 ml distilled water).

T**ble-2: Composition of gelatine nutrient agar (100ml)**

Constituents	Gram / 100 mL
Gelatin	1. 5
Glucose	0.1
Peptone	0.5
K_2HPO_4	0.2
Agar powder	1.5

3.3 Preparation of hippuric acid calibration curve

Ten ml of hippuric acid (HA) standard solution was prepared by adding HA (0.4 mg/ml), NaCl (300 mM), Sodium borate buffer (100 mM). From this eight solutions having different concentrations of hippuric acid ranging from 0.02 mg/ml to 0.16 mg/ml were prepared (Table-3).

Table-3: Concentration of HA sample and SBB solution

HA sample (mg/ml)	HA stock solution (µl)	Sodium borate buffer (SBB) (µl)
0.02	25	475
0.04	50	450
0.06	75	425
0.08	100	400
0.10	125	375
0.12	150	350
0.14	175	325
0.16	200	300

Nine test tubes (5 ml) were taken; one was for blank another 8 for different concentrations of hippuric acid. 500 µl of HA, different concentrations in each tube, was taken and 600 µl of quinoline were added to each of them followed by vortexing. Then 200 µl of benzene sulfonyl chloride (BSC) was added and mixed properly followed by incubation at 30ºC for 30 min. 3700 µl of ethanol was added and incubated again for 30 min at 30 ºC in dark. Then absorbance was measured at 492 nm and the calibration curve was obtained by plotting absorbance at 492 nm against HA concentration.

3.4. ACE inhibitory analysis and protein estimation by nanodrop.

All the isolates were separately inoculated in MRS broth containing 10% skim milk powder and incubated at 37ºC for 48 hours, then centrifuged at 7000 rpm for 20 min at 5ºC. Crude cell free extracts were collected and protein concentration was estimated using nanodrop. ACE inhibitory (ACEI) assay was done by direct spectrophotometric method (table-4) [Le et al., 2004].

Table -4: ACEI assay protocol

Components	**A (captopril,OC1-OC5)**	**B (control)**	**C (blank)**
100 mU/ml ACE (µl)	10	10	10
1 M HCl (µl)	0	0	100
ACEI (sample) (µl)	20	0	20
Incubate at 37°C for 30 minutes			
5 mM HHL (µl)	50	50	50
Incubate at 37°C for 30 minutes			
1 M HCl (µl)	100	100	0
ACEI (sample) (µl)	0	20	0

100 mM sodium borate buffer (µl)	320	320	320
Quinoline (µl)	600	600	600
BSC (µl	200	200	200
Incubate at 30°C for 30 minutes in dark			
Ethanol (µl)	3700	3700	3700
Incubate at 30°C for 30 minutes in dark			

ACE cleaves HHL into hippuric acid (HA) and histidyl leucine (HL). For each assay, a sample solution of ACE inhibitor (20 µl) and positive test control captopril (20 µl) were added to 10 µl of the ACE and incubated for 30 min at 37ºC, then 50 µl of 5 mM HHL in 100 mM sodium borate buffer (pH 8.3) containing 300mM NaCl was added followed by incubation at 37 ºC for 30 min. The reaction was stopped by adding 100 µl of 1 M HCl. Then Sodium borate buffer was added to the reaction mixture to make total volume of 0.5 ml in order to maintain the pH. Then 600 µl of quinoline and 200 µl of BSC were added to all the tubes followed by incubation at 30ºC for 30 minutes in dark. Finally 3700 µl of ethanol was added to all the tubes and incubation was repeated at 30ºC for 30 minutes in dark. Then the absorbance was measured at 492nm. The extent of inhibition was then calculated as follows:

$$\text{ACE inhibitory activity (\%)} = (B - A)/(B - C) \times 100$$

3.5. Development of watermelon ravioli

Overnight cultures of the selected bacteria were grown in MRS broth. Then centrifuged at 7000 rpm for 20 min at 5ºC and the pellets were re-suspended in sterile distilled water. 1 ml of each bacterial suspension was added to 100 ml of pasteurized milk and incubated for 5 hours. Then equal volume of sodium alginate (3%) was added and the mixture was added drop wise into sterile calcium chloride solution (0.5 M) forming beads containing bioactive peptides of milk. The beads were washed twice in water and then added to 100 ml sterile

watermelon juice, to which equal volume of sodium alginate (3%) was added. The mixture was added spoonful to calcium chloride solution (0.5 M) forming ravioli which were finally washed & refrigerated.

RESULTS AND DISCUSSION

1. Isolation of lactic acid bacteria from OMFED curd

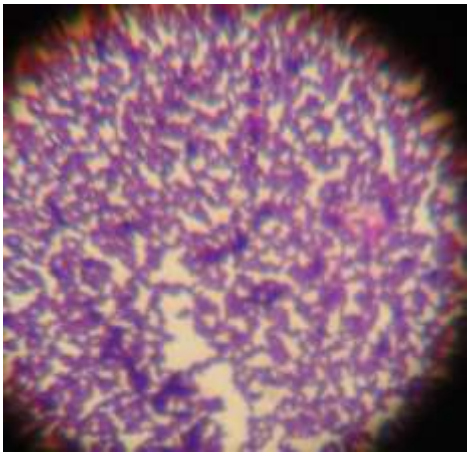


Fig-2 : OC1

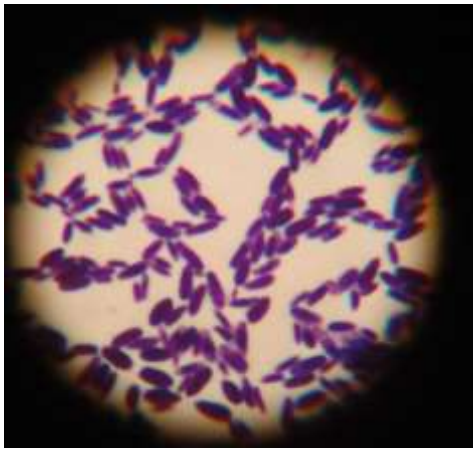


Fig-3 : OC2

Fig-4 : OC3

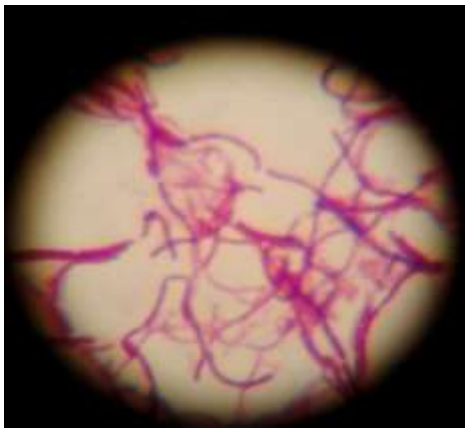


Fig-5 : OC4

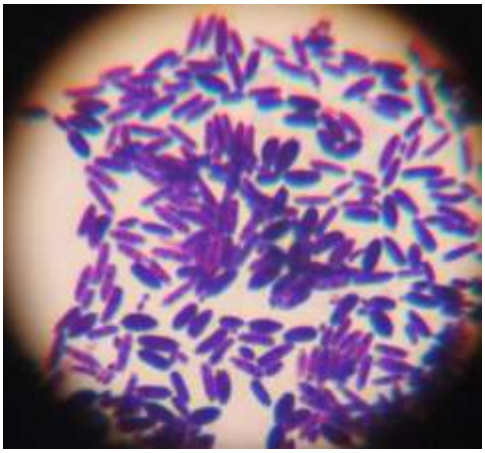


Fig -6 : OC5

OC1, OC2, OC3, OC4 and OC5 bacterial strains were isolated from OMFED curd, Rourkela (figures- 2, 3, 4, 5, 6).

2. Morphological characterization by gram-staining method

All the isolated organisms were bacteria. Except OC4 (OMFED CURD 4), all other bacterial isolates were gram positive (Table 5).

Table -5: Morphological characterization of isolates

Isolates	shape	Type of bacteria
OC1	Long rod	Gram positive
OC2	Long rod	Gram positive
OC3	Long rod	Gram positive
OC4	Long rod	Gram negative
OC5	Long rod	Gram positive

3. Screening for the proteolytic activity

Fig-7 OC1

Fig-8 OC2

Fig-9 OC3

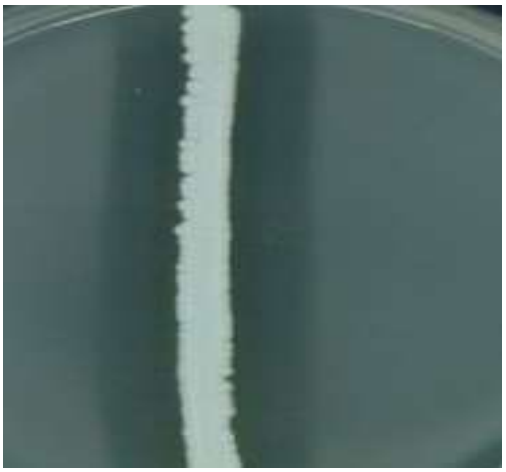


Fig-10 OC4

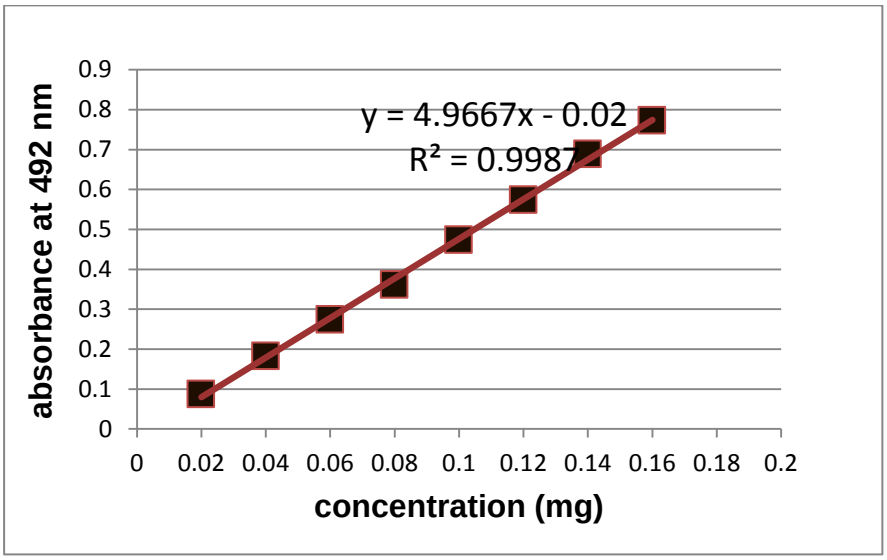
Fig-11 OC5

The isolated bacteria having proteolytic activity were able to break the protein present in gelatine nutrient agar media into peptides depending on their level of activity. After adding mercuric chloride the un-hydrolyzed gelatine was found to bind with mercuric chloride, as a result clear zones of gelatine hydrolysis were seen. The clearest zone was seen in case of OC1 followed by OC5, OC4 and OC3. The least clear zone was seen in OC2 plate. Hence, OC1 has highest and OC2 has lowest proteolytic activity while the rest three have intermediate activity levels.

4. Hippuric acid calibration curve

Considering the concentrations ranging from 0.02 to 0.16 mg of hippuric acid (HA), the standard curve was prepared by plotting absorbance at 492 nm against concentration of HA (Fig. 12).

Fig-12: Hippuric acid (HA) calibration curve showing absorbance at 492 nm



5. Protein estimation using nanodrop -

Control (un-fermented) showed more amount of total protein than the fermented samples (table-6).

Table-6: Estimation of protein concentration by nanodrop

Sample	Concentration (mg/ml)
control	66
OC1	45
OC2	61
OC3	60
OC4	50
OC5	47

6. Observation and calculation for ACEI assay

ACE inhibitory activity was calculated by formula:

ACE inhibitory activity (%) = (B-A) / (B-C) *100

Where, B=control

A=sample

C= blank

Isolate OC1 showed highest ACE inhibitory activity as it released less concentration of HA by the cleavage of HHL in presence of ACE. Hence less amount of the chromogen complex was formed by reaction of HA with quinoline and BSC, thus giving lowest OD. The ACEI activity of peptides produced from fermentation by OF3 was even found to be more than that of the commercial drug Captopril generally used for the treatment of hypertension. The other effective and comparable ACEI inhibitory peptide forming organisms were OF5, OF4 and OF3 (table-7). Hence these four organisms were selected for further use.

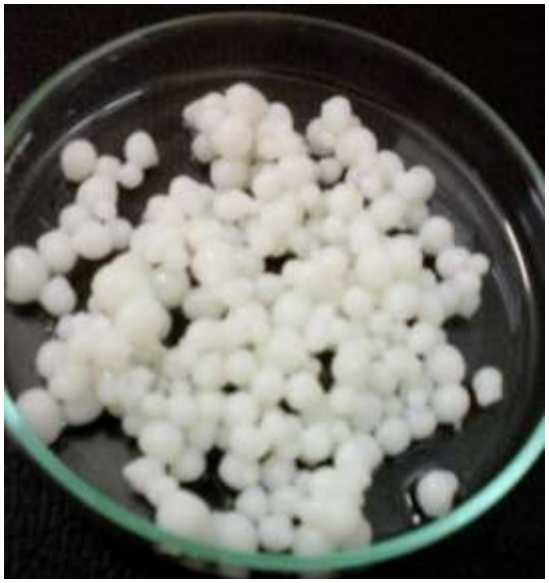
Table – 7: ACE inhibitory activity with respect to HA concentration

Sample	OD	ACEI (%)	HA concentration (mg/ 5ml)
control	0.071	0	0.0183
captopril	0.011	84.5	0.0062
OC1	0.005	93	0.005
OC2	0.064	10	0.017
OC3	0.041	42	0.0123
OC4	0.023	68	0.0087
OC5	0.017	76	0.0075

7. Development of watermelon ravioli having antihypertensive peptides

After 5 hours of incubation of the organisms in pasteurised milk, fermented and having antihypertensive peptides, encapsulation was carried out giving rise to small beads of the fermented milk (fig-13).

Fig-13: Fermented Omfed milk encapsulated into beads having antihypertensive peptides



Sterilised watermelon juice was combined with the prepared beads (fig-14) and after another phase of encapsulation into larger forms, the desired product, watermelon ravioli was formed (fig-15).

Fig-14 : watermelon juice combined with beads

Fig-15: Watermelon ravioli with antihypertensive bioactive peptides from fermented OMFED milk in presence of isolated bacteria.

CONCLUSION

Among the isolates, OC1 was found to have highest ACEI activity (93%) while OC5 (76%), OC3 (68%) and OC4 (42%) had moderate ACEI activity. OC2 (10%) was found to have least ACEI activity. So it can be concluded that in case of OC2 the ACE activity was not inhibited by the peptides formed, hence ACE acted on HHL (substrate) giving rise to high concentration of HA and histidyl leucine. The concentration of HA is thereby inversely proportional to the ACEI activity of the peptides formed after fermentation with respective isolate. The final product, watermelon ravioli has the ACEI activity due to the peptides as well as the nutritional quality of the fruit in a much more attractive and palatable form.

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