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STUDY OF ANTAGONISTIC PROPERTIES OF BACTERIA FROM FERMENTED PULSES BY REAL-TIME SURFACE PLASMA RESONANCE BIOSENSOR (BIAcore)

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ABSTRACT

Probiotics are functional food natural or processed contains known live microorganism or biologically active compounds which confer multiple health benefits on the host. Probiotics helps in prevention, management and treatment of chronic diseases. Foodborne bacteria confer probiotics that produce antimicrobial substances which shows narrow spectrum antagonistic properties against food spoilage bacteria. The antimicrobial compounds produced from foodborne bacteria are safe for human health and effective against pathogenic bacteria like *Listeria monocytogenes, Escherichia coli* and *Salmonella enterica*. Foodborne pathogens are among the most significant problems in maintaining the human health. The emergence of multidrug-resistant pathogens and the restriction on the use of antibiotics as growth inhibitors of the pathogens in the processed foods and feeds have drawn attention to the search for possible alternatives. Present work begins with the isolation of probiotics bacteria from fermented pulses. The organisms were grown in various media for enrichment of bacteriocin production. The culture's cell free supernatants were isolated from the bacteria for the antimicrobial screening by conventional Kirby-Bauer disc diffusion method and use of advanced real-time surface plasma resonance biosensor (BIAcore). Here we are presenting the results of the identification tests of the isolated bacteria and screening of bioactive compounds from culture supernatants binding with immobilized pathogens on BIAcore T200 instrument. The comparative studies prove to be reliable and more accurate than the conventional assay.

Key words: Antimicrobial activity, Bacteriocins, Kirby-Bauer disc diffusion, Surface Plasma Resonance Biosensor, BIA core T200.

INTRODUCTION

Probiotics are the "live microbial culture or living microorganisms which beneficially influence the health and nutrition when ingested in appropriate concentration exert health benefits beyond inherent basic nutrition for the host [1]. They are also widely used in clinical nutrition and complementary alternative medicine. Experts at the National Institutes of Health note that probiotics show some promise in the treatment of diarrhea, irritable bowel syndrome, vaginal infections, tooth decay and skin disease. The gastrointestinal tract of mammals hosts a high and diverse number of different microorganisms, known as intestinal microbiota. Many probiotics were originally isolated from the gastrointestinal tract, and they were defined by the Food and Agriculture Organization of the United Nations (FAO)/WHO as live microorganisms which when administered in adequate amounts confer a health benefit on the host.Probiotics exert their beneficial effects on the host through four main mechanisms: interference with potential pathogens, improvement of barrier function, immunomodulation and production of neurotransmitters, and their host targets vary from the resident microbiota to cellular components of the gut-brain axis. The beneficial effect of probiotics is mainly through their interaction with the intestinal microbiota and with the intestinal mucosa. During fermentation, lactic acid bacteria can produce a number of bioactive metabolites such as bacteriocins, biogenic amines, exopolysaccharides, and proteolytically released peptides [2]. To prepare a solid food probiotic matrix osmotic dehydration is carried out in which, food stuff is soaked in solution of low water activity where food water is partially removed and impregnated as food pieces that has twofold effect [3].

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Probiotic or food-grade bacteria capable of producing antimicrobial peptides can be isolated from different food sources such as fermented cereals /pulses. Cereals being rich source of prebiotics such as beta-glucan and arabinoxylan, galacto-, and fructo-oligosaccharides are considered for development of probiotic foods [4]. Pulses are good sources of bioactive compounds such as polyphenols, phytosterols and non-digestible carbohydrates that play important role in physiological and metabolic rates. Pulse fibres, resistant starch and oligosaccharides function as probiotics and possess several other health benefits such as anti-inflammatory, anti-tumour, and reduce glucose-lipid levels [5]. Pulses do provide nutrients and promotes the growth of microorganisms within considering a probiotic [6].

The selection of indicator organisms was carried out by concentrating on food spoilage and food pathogens that causes the major illness on human health. The indicator strains used were: *Escherichia coli, Listeria monocytogenes* and *Salmonella enteric* [7]. *Escherichia coli* is a Gram-negative, rod-shaped, facultatively anaerobic commensal bacterium which can also be an enteropathogenic or extraintestinal pathogen which causes gastro-enteritis in both humans and animals by producing toxins [8]. *Listeria monocytogenes* is a Gram-positive, rod-shaped, non spore-forming, motile, facultatively anaerobic, catalase-positive and oxidase-negative bacterium. The bacteria express a beta hemolysin, which causes destruction of red blood cells [9]. *Salmonella enterica* is a Gram-negative, rod-shaped, facultatively anaerobic bacterium which causes foodborne infectious diseases like typhoid fever and nontyphoid salmonellosis [10].

The screening for identification of bacteriocin producing bacteria was done conventionally by Kirby-Bauer disc diffusion method [11]. The evaluation of the effect of antimicrobial peptides or bacteriocins produced by the isolates were tested against the indicator organisms by the disc diffusion method [12].

An advance study was carried out to check the efficacy of the interaction between the protein and cell by advanced automated real-time biacore technique. Biacore is the label-free surface plasmon resonance (SPR) biosensor preferred strategy to examine protein-peptide interaction. Surface plasmon resonance (SPR) is one of the most commonly used techniques to study protein-protein interactions. The main advantage of SPR is it gives on the ability to measure the binding affinities and association/dissociation kinetics at varied concentration of complexes in real time, in a label-free environment, and using relatively in small quantities. The method is based on the immobilization of one of the binding partners, called the ligand, on a dedicated sensor surface called the chip. Immobilization is followed by the injection of the other partner, called the analyte, over the surface containing the ligand. The binding is monitored by subsequent changes in the refractive index of the medium close to the sensor surface upon injection of the analyte. The use of SPR to study the dynamic systems of protein secretions owing to its ability to detect both weak and strong interactions ranging from the millimolar to the nanomolar range has increased spectacularly. SPR can be used as a primary tool to screen interacting partners and also as a validation tool for interactions that is previously identified by conventional methods. The determination of the affinity or kinetics of an interaction, as can be done by SPR, is fundamental to understanding the nature of binding at the cellular level. In the SPR biosensor, the analysis of interaction between the molecules is sequelled numerically and compared with other protein-protein binding assays to determine the priority affinity binding partner [13]. Bacteriocins are ribosomally synthesized, proteinaceous compounds that inhibit the growth of closely related bacteria. Even within the subcategory of bacteriocins, the peptides vary significantly in terms of the gene cluster responsible for expression, and chemical and structural composition. The polycistronic gene cluster generally includes a structural gene and various combinations of immunity, secretion, and regulatory genes and modifying enzymes. Chemical variation can exist in amino acid identity, chain length, secondary and tertiary structural features, as well as specificity of active sites. This diversity posits bacteriocins as potential antimicrobial agents with a range of functions and applications. Those produced by food-grade bacteria and applied in normally occurring concentrations can be used as GRAS-status food additives. Bacteriocins act as an alternative to antibiotics which wide range of action on antibiotic resistant pathogens and nosocomial infectious organisms. Use of microcins is a possible alternative to control gram negative bacteria [14].

MATERIALS AND METHODS

Screening of non-lactic acid bacteria from different food samples

It is common knowledge that microorganisms, bacteria have capabilities to produce a huge amount of bioactive antimicrobial compounds. Pulses like Soya bean (*Glycine max* L.Merril), Desi/ Black chana (*Vigna mungo* L.Hepper) and Kabuli chana (*Cicer arietinum* L.) were used for isolation of bacteria. Fermented chunks were made from fermented mashed pulse like Soya bean (*Glycine max*), Desi/ Black chana (*Vigna mungo*), and Kabuli chana (*Cicer arietinum*) by soaking them in water, grinding and finally drying the small chunks in the sun. For isolation of bacteria, fermented material was soaked in 10 ml of distilled water at 27°C for 15mins. After crushing in sterilized mortar with pestle, it was serially diluted in sterile distilled water in the range from 10^{-1} to 10^{-7}. Isolation of bacteria was carried out for the dilutions from 10^{-5} to 10^{-7} by pour plate method on a media commonly used for the isolation of non-lactic acid bacteria, nutrient agar and incubated at 37°C for 24-48hrs [15].

Procurement of indicator organisms

Pure cultures of food spoilage bacteria were procured from Microbial Type Culture Collection (MTCC) and Gene Bank, Chandigarh, India and maintained in the table with their respective MTCC numbers in their respective media as instructed.

All the organisms were revived and tested in nutrient broth and nutrient agar. The organisms are maintained as per the MTCC standard instructions.

Screening

Preparation of cell free supernatants

The isolated bacterial strains were grown in Nutrient Broth (NB), incubated at 30°C for 48 hrs in shaking condition at 120 rpm. The cultures were centrifuged at 10000 g for 15 min. The supernatant collected was filtered with 0.45µM Whatman No.1 filter membrane to get cell free supernatant [16]. Through the statistical tools studied, the maintenance of aerobic condition with constant supply of oxygen plays a crucial role in the growth of non-lactic acid bacteria and in the production of bacteriocin [16].

Preparation of inoculum

The evaluation of antimicrobial activity reckoned by preparing 24hrs fresh culture of indicator bacteria by adjusting the turbidity of cell density with the spectrophotometer to the transmittance to produce absorbance of 0.08-0.1 that correlates to 0.5 McFarland standard at 625 nm wavelength. This procedure yielded the cell stock suspension of 1 x 10^7 to 10^8 colony forming units (CFU) per mL [14].

***In vitro* antimicrobial assay by Kirby-Bauer disc diffusion method**

A volume of 100 µl of inoculum suspension previously adjusted to 0.5 McFarland standard of each indicator bacteria was swabbed evenly on prepoured sterilized nutrient agar plates set for the disc diffusion assay. The lid was left ajar to allow the absorption of excess surface moisture for 10mins before placing the sterile discs. Sterile 5 mm diameter discs were prepared using Whatmann No. 1 filter paper and placed on bacteria swabbed nutrient agar plates equidistantly round the margin of Petridish. 10 µl of cell free supernatant from nutrient broth were dispensed on the disc. The plates were incubated at 37°C for 24 hrs. At the end of the incubation period, the antagonistic activity was recorded as the width of the clear inhibition zone formed around the disc (diameter of inhibition zone plus diameter of the disc). The values are the means of three independent experiment performed in triplicate [17].

Statistical analysis

Statistical analysis was performed using SPSS software: Version 20.0. The results were expressed as mean ± SD (n=3). Experimental data were analysed using multivariate ANOVA depending on the nature of data set. Means were separated by post hoc analysis – Tukey HSD with the level of significance at $P<0.05$.

Advanced automated real time Biacore technique

Real-time, label-free detection of functionalization of antimicrobial peptide present in cell free supernatant with high selectivity and sensitivity for the indicator organism is demonstrated using an interdigitated impedimetric array called the Biacore technique. Biomolecular discrimination is one of the most important ways to discriminate closely related species which can be made easy, versatile and reliable by using different sensing systems and one among is the Biacore-carboxymethylated dextran coated sensor chips.

Surface Plasmon resonance (SPR)

The surface plasmon resonance (SPR) biosensor is a useful tool to analyze numerically the interaction of certain molecules. The most important advantage of the SPR assay as compared with other protein-protein binding affinities and association/dissociation kinetics of complexes in real time, in a label-free environment, and using relatively small quantities of materials and can calculate the affinity between protein and its binding partner. The method is based on the immobilization of one of the binding partner- indicator organism at 0.5 McFarland standard, called the ligand, on a dedicated sensor surface. Immobilization is followed by the injection of the other partner- cell free supernatant, called the analyte, over the surface containing the ligand. The binding is monitored by subsequent changes in the refractive index of the medium close to the sensor surface upon injection of the analyte and a sensogram is produced as a representation of its binding kinetics [18].

SPR interaction analyses were performed using a Biacore T200 optical biosensor (GE Healthcare Life Sciences, Bangalore, India). Biacore T200 is a molecular interaction analysis system that delivers the highest quality data for every interaction parameter.

The detailed protocol includes;

The immobilization of indicator organism

The indicator organism is immobilized onto the CM5 (Carboxy Methylated) gold microelectrode chip using amine coupling. Amine coupling is the most applicable covalent coupling chemistry used to immobilize protein ligands. The dextran matrix is activated with a 1:1 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.1 M N-hydroxy succinimide (NHS) to create reactive succinimide esters. For immobilization, the free carboxyl groups of the amino acid residues were activated with EDC/NHS then reacted with the ethylenediamine-derivatized carboxy methyldextran sensor chip to obtain the desired ligand concentrations. The standard activation time required to reach the immobilization level is 7mins [19]. The ligand is then injected in low salt buffer lacking primary amines at a low pH below the isoelectric point of

the protein. Under these conditions, the ligand acquires a positive charge and effectively preconcentrated into the negatively charged carboxymethyl dextran matrix. The unreacted esters are blocked with ethanolamine. This acts as a Baseline which monitors the immobilization level.

Analyte- ligand binding analysis

SPR measurements were carried out in phosphate buffer saline (0.1 M phosphate buffer with 27 mM KCl and 1.37 M NaCl 0.005% polysorbate 20 pH 7.4). Onto the immobilized ligand on the dextran matrix that acts as a substrate film to which the analyte, cell free supernatant to be detected attaches through hydrophilic binding to form a positive interaction, else a negative interaction. SPR causes reduction in the intensity/ density of light at a specific angle from the glass slide of the sensor chip CM5. Nisin at the concentration of 1ppm (1µg/mL) is used as a positive control and nutrient broth as negative control.

Data analysis

Data was collected with the Biacore control software version 3.0. Experiments were performed by monitoring the refractive index changes as a function of time under a constant flow rate of 45µl/min. The relative amount of active compound bound to the indicator organism was determined by measuring the net increase in refractive index over time compared to control running buffer. There is an inline subtraction of reference surface during the run. The result from the detection of the change in RI is displayed as a sensogram plotted with the binding response (RU) on Y-axis against time on X-axis. The representation of sensogram can be either curve graph or a bar chart graph.

RESULTS AND DISCUSSION

Selection of colonies

The colonies which are opaque, moderately larger in size, cream/white coloured, prominent surface texture and shape were isolated and dispensed into nutrient broth. 59 colonies with different morphological features were isolated which was considered primarily as a single isolate of which 26 isolates from soya bean (SB), 12 isolates from black chana (BC), 21 isolates from kabuli chana (KC).

***In vitro* antimicrobial assay by Kirby-Bauer disc diffusion method**

Out of 59 isolates 3were –ve cocci, 8 were –ve coccobacilli, 13 were +ve cocci, 13 were +ve coccobacilli, and 22 were +ve bacilli. During primary screening against the indicator organisms *E.coli, L.monocytogegens* & *S.enterica* showed positive growth in control plates and the zone of inhibition was seen prominentally by 15 isolates from pulses against *E.coli.* Repeated experiments were conducted for further screening in triplicates.

On consecutive screening of cell free supernatants of the isolates from pulses by Kirby-Bauer disc diffusion method revealed that 6 isolates exhibited positive antagonistic activity against *E.coli* (10.16%) and 3 isolates against *L.monocytogenes* (5.08%). The zone of inhibition varied from highest 10 mm to the lowest 5.66 mm. 5 mm showed no zone of inhibition which corresponded to negative activity. During the conventional procedure of antimicrobial assay none of the isolates showed any antagonistic activity against *S.enterica*.

Advanced automated real time Biacore technique

The potential isolates exhibiting significant bacteriocin activity were selected which resulted in the antagonistic activity against all the three indicator organism viz., *E.coli*, *L.monocytogenes* and *S.enterica*. SPR Biacore T200 showed positive sensogram were plotted for the activity of cell free supernatant that ranged from 0.5 to 18. The buffers, positive and the negative control did not exhibit any activity. The details are tabulated and represented in the bar graph.

The screening of non-lactic acid bacterial isolates for the production of bacteriocin or bacteriocinic peptides were carried out by the antagonistic assay against potential food pathogens. The results of conventional Kirby-bauer disc diffusion method revealed the antimicrobial effect of cell free supernatants prominentally against *E.coli*, partially against *L.monocytogenes* and negative activity against *S.enterica*. On the other hand, the use of advance surface plasma resonance biosensor seemed to be promising in exhibiting the cell protein- protein interaction in the form of bar graphs. The comparative investigation finally concluded the advantages in the use of biacore for the study involving the specific protein interaction during antagonistic activity between the cell and the peptide secreted over the use of conventional disc diffusion method.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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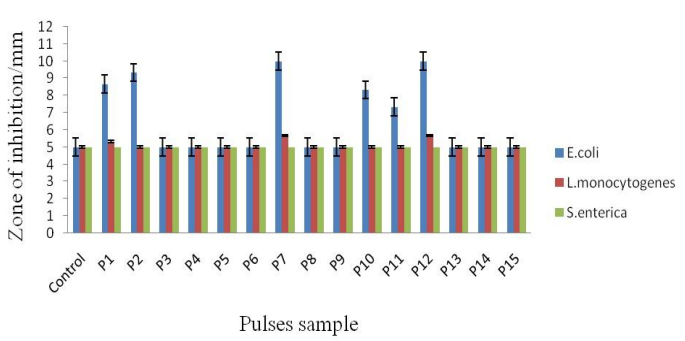
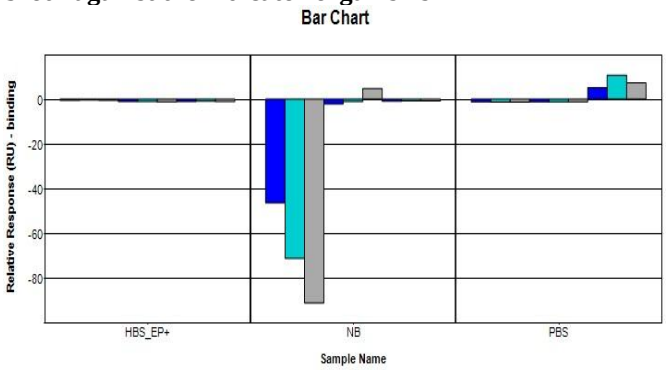
Table 1. MTCC organisms with their number

SL NO	ORGANISM	MTCC NUMBER
1.	*Escherichia coli*	10312
2.	*Listeria monocytogenes*	1143
3.	*Salmonella enterica*	1164

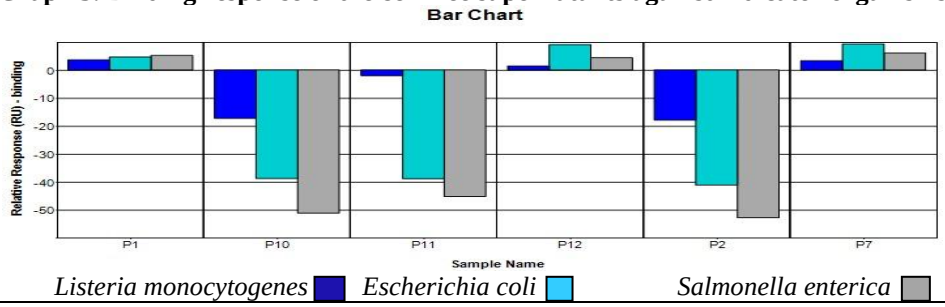
Graph 1. Antagonistic activity of isolates from fermented pulses by Kirby-Bauer disc diffusion method



Graph 2. Binding response of the buffers and nutrient broth against the indicator organisms



Graph 3. Binding response of the cell free supernatants against indicator organisms



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