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ORIGINAL ARTICLE

Isolation of A Novel Bacterial Strain Planococcus Plakortidi from Ross Broiler Chicken Gut (CECUM) with Combination of Non-Starch Polysaccride Degrading Pectinase Enzyme

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ABSTRACT

Purpose: In many countries, including Pakistan, broiler chickens and the poultry industry are severely affected by bacteria. Broiler chickens are infected with Gram-positive and Gram-negative bacteria.

Methods & Findings: The current study successfully isolated non-starch polysaccharide (NSP) degrading a novel strain Planococcusplakortidi (PCB2) from the chicken gut cecum, screened out from 60 samples.

Practical Implication & Conclusion: Furthermore, the isolate was taxonomically characterized based on its cellular morphology, physiological, and chemotaxonomic characteristics, as well as the phylogenetic similarity index of the 16S rRNA gene sequence. The bacterium was found to exhibit maximum production of pectinase enzyme on the eighth day of incubation at 37℃.

Keywords: Broiler chicken, gut microbiota, cecal bacterial diversity, chicken food digestion, Non-starch Polysaccharides

INTRODUCTION

The intestinal bacteria in farm animals have long been of research interest because of their impact on the health and well-being of the host animals, which is relevant to the efficiency of livestock production, as well as the quality and safety of their products[1-2]. Humans need chicken meat for various reasons, including its rich protein, trace elements, and fat. There has been an increased interest in free-range and organic chicken meat as the standard of living rises[3-4]. Food safety and quality have rapidly become global concerns due to an increasingly international population[5-6]. The broiler chicken is one of the most farmed animals in the world due to its important role as a protein source for humans[7] and as a powerful experimental model in both fundamental and applied research[6-8].

The local populations of free-range (domestic/native) chicken breeds are well-adapted to harsh environmental conditions and can be lengthy set up in rural areas[9-10]. Over 80% of rural areas and poor households in developing countries rely on domestic chickens for meat and eggs[11] Free-range (slow-growing/native) chickens may not show the same genetic profiles of their growth traits as broiler chickens[12]. It has been reported in the literature that the strain of the chicken affects feed consumption, digestibility, feed conversion ratio, and growth rate over time[11-13] . Researchers studying indigenous chickens in many parts of Africa discovered high genetic variability within ecotype populations[14-15], indicating that these chickens could be genetically improved through selective breeding.

Non-starch polysaccharides (NSP) can be fermented by beneficial microorganisms living in the poultry intestinal tract resulting in improved microbiota composition and production of short-chain fatty acids, alongside reduced competition between the microbiota and host for valuable nutrients[16]. While broilers are very sensitive to dietary NSP content and composition, the degradation process is very low and limited at soluble friction[17-18] because NSP directly influences digesta passage rate, microbiome composition, and intestinal health. Therefore, NSP-degrading enzymes have become common in poultry diets due to their ability to improve nutrient utilization and productive performance, suggesting it warrants consideration during feed formulation. Modern broiler diets contain approximately 10 to 12% of total NSP[19]. This NSP is categorized based on whether it is soluble or insoluble in water, with both inducing advantageous and detrimental effects on the poultry's gastrointestinal tract. Soluble NSP has a high water holding capacity, causing increased digesta viscosity when in excess. The consequence of this is reduced accessibility of enzymes to substrates and reduced absorption of nutrients through the gastrointestinal wall, as well as increased water consumption and, thus, excreta moisture content[18].

Pectinase production occupies about 10% of the overall manufacturing of enzyme preparations. Pectinolytic enzymes are widely used in the food industry for juice and wine production[20]. However, the NSP-degrading pectinase enzyme is subsequently improving the nutrient digestibility and absorption in the chicken gut cecum[21]. Supplementing diets with pectinase enzymes allows for reducing the anti-nutritive effects of NSP[22].

Planococcus plakortidis, one of the nine species of the genus Planococcus, was first time isolated from a marine sponge [Plakortis simplex (Schulze)], which was collected by a scuba-diving team from Clearance and Collection Divers of the Indian Navy, at a depth of 30 m in the Bay of Bengal, off the coast of Gopalpur in the Indian state of Orissa[23].

The chicken gut bacterial community had been limited to identification through culture-dependent techniques (culture-based)[24]. Furthermore, the ready detection of cultivated bacteria through this technique is limited, and the numbers still need to be higher due to their unknown nutritional requirements[25-26]. According to Kohl (2012)[27], it is far anticipated that about 99% of bacterial species can not presently be grown under laboratory conditions. Those limitations triumph over through the advancement of culture-independent and moleculartechniquessuitable for the analysis of microbes[28] .

These new strategies also help distinguish among chickens' growing enteric health issues. Studies using these techniques are based on direct bacterial DNA extraction from the sample of chicken gut and the taxonomically steady sequence of genes. Through culture-independent techniques, researchers have identified many more bacteria from chicken intestines than formerly identified based on totally culture-dependent methods[28-29].

Since the advancement of PCR, it has been utilized for discovering specific microbes, characterization of functional genes, and sequencing[26]. Metagenomic is an analysis sequencing-based technique that uses high-throughput, non-specific bacterial DNA sequencing to record genes and permits scientists to evaluate functional diversity. Analysis of mycobacterial diversity within the chicken cecal microbiome was conducted based on comparative metagenomic pyrosequencing[30]and analyzing the function of the chicken cecal microbiota based on metagenomic sequencing and analysis[31]. Metagenomics can identify potential features but not the actual functions of microbial communities[27-32].

The advantage of microbial culture-independent technologies includes being able to: increase our understanding of the function and composition of gut microbiota, identify a mutation in microorganisms by quickly enumerating and identifying the presence of a wide variety of bacteria, enhance knowledge of the

interactions between hosts and the microbiota[29-33], and assist in advancing the selection of different products which boost gut health[34]. Interpretation of microbial analyses and the use of these advanced molecular techniques may be complicated due to interactions which can also exist between resident microorganisms and the host. The diet composition may also impact the microbial community[35]. Moreover, currently, most researchers have the potential to subculture some bacteria and the ability for microbial groups to escape detection, an underestimation of microbial numbers and diversity can also result[25-35].

MATERIALS & METHODS

Experiment Design: The experiment was designed according to Nadeem et al. (2005)[36] at the animal house Department of Zoology, Shah Abdul Latif University, Khairpur. One-day-old Ross broiler chicks weighing approximately 40g to 50g were purchased and separated randomly into six sets, each consisting of ten chicks in four sq ft[37].

Three sets were under experiment with simultaneously with random design. Each set of broiler chicks was maintained separately in pens roughly five feet long and three feet wide. All chicken sets were decontaminated before the experiment began. During the experiment, 3-to-4-inch sawdust was employed as waste and litter in each set. It was regularly mixed to keep it dry. To prepare for the experiment, all the broiler chicks received the recommended Pakistani schedule of vaccinations against infectious bronchitis, bursal disease, Newcastle disease, hydropericardium syndrome, and other respiratory illnesses. Fresh water and mashed food were regularly provided for up to 42 days throughout the experiment.

Sample Collection: The chickens were transported from the animal house to Post Graduate Research Laboratory (PGRL), Institute of Microbiology, Shah Abdul Latif University, Khairpur, for the collection of gut samples, and after dissection of chicken gut samples (1g) were collected from the major section cecum (approximately 3 cm long) of chicken gut.

Sample Processing for isolation: The collected sample was inoculated in a vincent broth containing Pectin as a carbon source to isolate Pectinolytic bacteria. The culture was incubated for ten days in a shaking incubator at 37℃ (160rpm). Optical density (OD) reading was monitored after a one-day interval up to 10 days incubation period. However, the highest OD sample was plated on a vincent agar medium containing 1g of Pectin as a carbon source and incubated for 24 hours at 37℃. After the incubation period, growth was observed based on the colonial characterization of pectinase-degrading bacteria. The selected colonies were transferred onto freshly prepared nutrient agar for pure culture studies and further identification.

Biodegradation of Pectin: The shake flasks containing vincent broth and 1% of Pectin as the sole energy source were inoculated with freshly grown bacterial inoculum having growth at a ratio of (1.5×108 CFU/ml), and biodegradation experiments were run for 120 hours at 37ºC under agitation (160rpm). The bacterial growth kinetics were recorded every 24 hours, including zero hours of incubation, by taking growth absorbance at 600nm using a spectrophotometer (Jennway 6300, Germany). Control experiments were run without inoculums under similar experimental conditions. All the experiments were run in triplicate, and data records were presented as the mean of three values ± standard deviation—the per cent removal of Pectin was determined using UV-Vis Spectrophotometerat 600 nm.

Production of Pectinase Enzyme: To evaluate the production of pectinase enzyme, freshly grown bacterial cells (1.5×108 CFU/ml) were inoculated into the 250ml conical flasks containing vincentbroth+pectin and incubated in a shaker incubator at 37℃ (160rpm) for ten days; all experiments were carried out in triplicate. During the incubation period one day interval, a 3ml sample was transferred into sterilized tubes for enzyme activity analysis. These tubes were kept at four °C in the PGRL, Institute of Microbiology.

Pectinase activity was determined using a dinitrosalicylic acid reagent (DNS) method by measuring reducing sugars released after the enzymatic reaction; for this, Glucose was used as a standard. The assay was carried out according to the modified method[38]. 1 ml of cell-free culture supernatant was added to 0.2 ml substrate solution containing 1% pectin prepared in 2 ml 1M sodium citrate buffer of pH 7.0. The reaction mixture was incubated at 37°C for 25 minutes. The reaction mixture was stopped by adding 3 ml DNS reagent and a boiling water bath for 15 minutes. After cooling, the mixture was centrifuged at 5000rpm for 5 minutes, and 2 ml of the supernatant was transferred into a new test tube. The absorbance was measured at 550nm using a UV spectrophotometer.

Optimization of pectinase enzyme

Effect of temperature on pectinase production: A 100 ml production medium was prepared, sterilized and 1 ml inoculum was added and then incubated at different temperatures at 27°C, 32°C, 37°C, 42°C, and 47°C, after which culture filtrate was harvested every 24 h. The enzyme activity were studied for up to 5 days.

Effect of pH on pectinase production: A 100 ml production medium was prepared, and the pH of the medium was adjusted to 6.0, 6.5, 7.0, 7.5, and 8.0. The sterilized production media were inoculated with 1 ml suspension and incubated under shaking conditions. The enzyme activity and protein content study were done every day for up to 5 days.

Identification of Bacterial Isolates: The cultural characteristics were recorded on the surface of nutrient agar. The isolates were studied for their colonial, morphological, and biochemical characteristics. The tests were performed for the morphologic characteristics, including gram staining, spore staining, capsule staining, and motility test. The Indole, methyl red, Voges Proskauer, citrate, urease, nitrate reduction, catalase, and oxidase tests were studied for their biochemical characteristics. Sugar fermentation tests (i.e.lactose, mannitol, Glucose, and sucrose fermentation) were performed using nutrient broth (1% sugar), and results were recorded.

Molecular identification using 16S rRNA: For molecular identification, universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used for the partial sequencing of the 16S rRNA at the selected bacterial isolates, at Macrogen Inc., Seoul Korea. Following purification, sequences were acquired from the amplified gene products using the universal primers for sequencing i.e.

800R (5'-TACCAGGGTATCTAATCC-3') and 518F (5'-CCAGCAGCCGCGGTAATACG-3') and ABI PRISM Big DyeTM Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystem, USA).

Phylogenetic correlation analysis: The Basic Local Alignment Search Tool (BLAST) program analyzed phylogenetic correlation. NCBI database was used to compare the partial 16SrRNA gene sequences determined in this study to determine the similarities in the gene sequence. The Maximum Likelihood Method was used to infer the evolutionary history. MEGA6 was used to carry out the evolutionary analyses, following Tamura et al. (2004)[39].

RESULTS & DISCUSSION

The isolated bacterial species with significant results were identified through 16S rRNA gene amplification and sequencing methods. A close correlation between gut-microflora and the growth performance of broiler chicken was made using molecular approaches and bioinformatics tools to disclose the secret behind the excellent meat quality of broiler chicken[40].

To make the best isolation from broiler chicken gut, a minimal salt medium was prepared with non-starch. Polysaccharides (Pectin) were used with control. Medium inoculated samples taken from the chicken gut (cecum) were incubated in a shaker incubator for ten days at 37℃, a day after optical density (OD) was measured (Figure-1 and Table-1).

The highest growth was measured with pectin on the eighth day; 1ml inoculum from each sample was transferred onto nutrient agar, spread with a spreader, and incubated at 37℃ for 24 hours. Second-day growth was observed for the microscopic and morphological identification gram staining; simple staining, shape, capsule staining, and motility tests were performed (Table 3). For further identification, biochemical tests, i.e. catalase, oxidase, indole, methyl red, Voges Proskauer, citrate utilization (IMViC), nitrate reduction, urease, and sugar fermentation tests (glucose, lactose, maltose, sucrose) for acid and gas production (Table-4). Molecular and bioinformatic approaches further characterized best-degrading bacteria.

This is the first study performed on broiler chicken gut in which Planococcus plakortidis bacterium was found to degrade the pectinase enzyme, which was first isolated from the chicken gut before it was isolated from a deep marine sponge near china[23]. However, some species from the genus Planococcus. They exhibit heavy-metal resistance and can degrade linear alkanes or aromatic hydrocarbons[1]. The Planococcus sp. S5 grows on salicylate or benzoate and produces a catechol 2, 3-dioxygenase that shows high reactivity toward 4-chlorocatechol. The haloalkaliphilic bacterium Planococcus sp. ZD22 can degrade benzene, toluene, xylene, and halogenated benzene and use them as the sole carbon sources[1]. Many studies have focused on the adaption mechanisms of Planococcus spp. under high salt environments or subzero conditions.Whereas, highest bacterial growth was studied on 8th day with OD 0.911 at 600nm (**Table & Figure.1**).

Table-1: Growth absorbance of chicken gut samples in vincent broth medium enriched with pectin as sole carbon and energy source.

Days	Control	Caecum
0day	0.231	0.188
2day	0.211	0.337
4day	0.299	0.651
6day	0.304	0.736
8day	0.311	0.911
10day	0.189	0.721

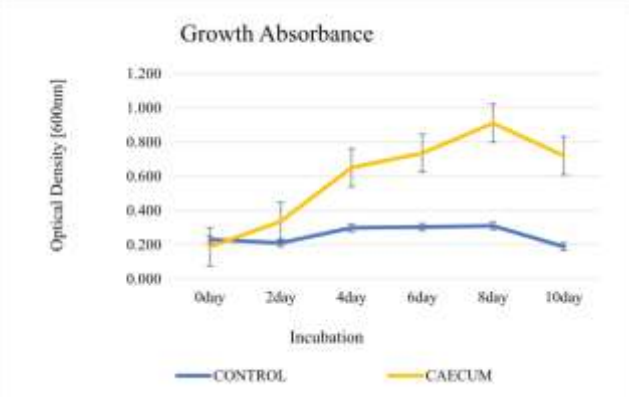

Figure-1: Growth absorbance of chicken gut samples in vincent broth medium enriched with pectin as sole carbon and energy source.

The vincent broth medium enriched with 1% pectin was inoculated with 1g chicken gut (cecum) sample and incubated in shaker at 37℃ (160rpm) upto 10 days. After oneday interval 3ml from inoculated broth, ample was taken to analyse bacterial growth (OD) using spectrophotometer. The experiment was performed in triplicate and results were recorded in comparison to the control (uninoculated broth). The maximum growth (O.D $_{600nm}$ 0.911) was recorded on the eight day of incubation with pectin enriched medium while the growth pattern in control was static as it was uninoculated broth.

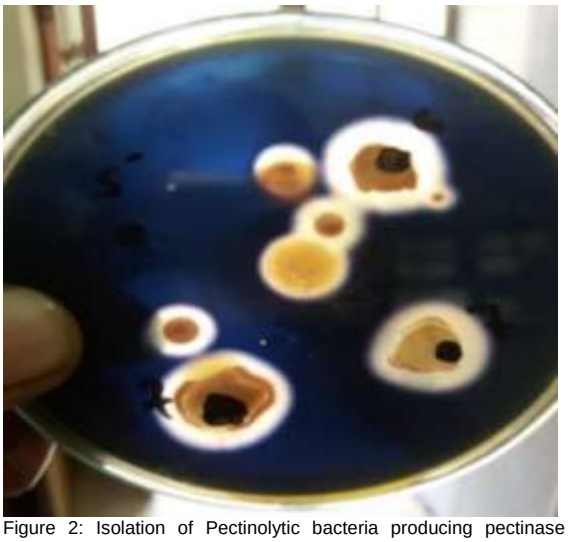

Figure 2: Isolation of Pectinolytic bacteria producing pectinase on the surface of Mineral salt medium (MSM) containing Pectin as sole carbon and energy source.

The collected growth absorbance sample was inoculated onto the surface of MSM agar containing pectin as sole carbon and energy source for the isolation of Pectinolytic bacteria. The clear zone of pectin hydrolysis was observed and after confirmation these pectinolytic bacteria were purified by repitative streak plate technique onto the surface of nutrient agar plate.

Table-2: Growth absorbance of pectinolytic bacteria (PCB2) in vincent broth medium enriched with 1% pectin as sole carbon and energy source.

Days	Control	PCB2
24hr	0.443	0.564
48hr	0.410	0.987
72hr	0.443	1.076
96hr	0.481	1.131
120hr	0.532	1.098

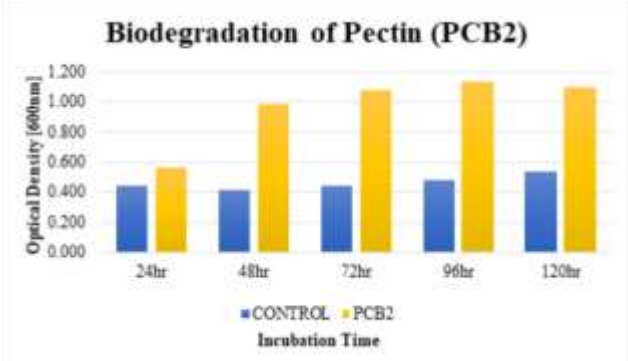

Figure-3: Growth absorbance of pectinolytic bacteria (PCB2) in vincent broth medium enriched with 1% pectin as sole carbon and energy source.

The vincent broth medium enriched with 1% pectin was inoculated with pure pectinolytic bacteria and incubated in shaker at 37℃ (160rpm) upto 5 days. After oneday interval 3ml from inoculated broth, sample was taken to analyse bacterial growth (OD) using spectrophotometer. The experiment was performed in triplicate and results were recorded in comparison to the control (uninoculated broth). The maximum growth (O.D $_{600nm}$ 1.131) was recorded on the 4th day of incubation (96 hours) with pectin enriched medium while the growth pattern in control was static as it was uninoculated broth.

Table-3: Quantitative Determination of pectinase enzyme using Elisa plate reader and Spectrophotometer at 550nm from the samples of pectin degradation experiment.

Spectrophotometer (550nm)		ELISA (550nm)	
Bacterial Isolate	OD	Bacterial Isolate	OD
Control	0.369	Control	3.813
PCB2	0.755	PCB2	4.813

A. Growth absorbance (O.D $_{550nm}$ 0.755) for the pectinase enzyme using spectrophotometer. B. Growth absorbance (O.D $_{550nm}$ 4.813) for the pectinase enzyme using Elisa plate reader.

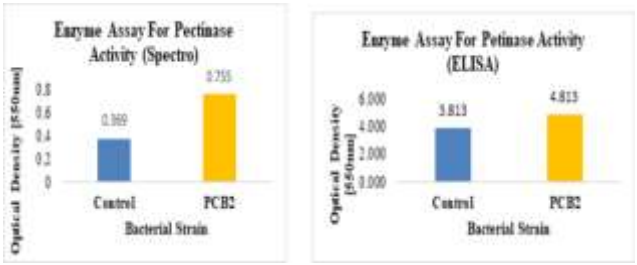

Figure-4: Quantitative Determination of pectinase enzyme using Elisa plate reader and Spectrophotometer at 550nm from the samples of pectin degradation experiment.

A. Growth absorbance (O.D $_{550nm}$ 0.755) for the pectinase enzyme using spectrophotometer. B. Growth absorbance (O.D $_{550nm}$ 4.813) for the pectinase enzyme using Elisa plate reader. The vincent broth medium enriched with 1% pectin was inoculated with pure pectinolytic bacteria and incubated in shaker at 37℃ (160rpm) upto 5 days. The maximum growth absorbance (O.D $_{550nm}$ 0.755 & 4.813) for the pectinase was determined using spectrophotometer and Elisa plate reader respectively.

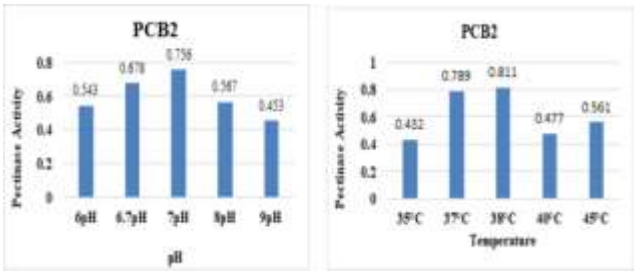

Figure-5: Quantitative determination of pectinase enzyme under optimized culture conditions.

A. Maximum pectinase production was recorded under optimized pH culture conditions at pH 7. B. Maximum pectinase production was recorded under optimized temperature culture conditions at temperature 38°C.

Table-4: The Morpho-microscopic characteristics of the chicken gut bacterial isolates

Isolates	Cultural Characteristics	Microscopy & Morphology of Isolates				
		GS	Shape	SS	CS	Motility
PCB2	Solid liquid Colonies are yellow orange in colour	+	Cocci/rods	-	-	+

Note: GS= Gram Staining, SS= Spore Staining, CS= Capsule Staining, +=Positive, -=Negative

Table-4: Biochemical identification and sugar fermentation tests of chicken gut bacterial isolates.

Isolates	C	O	NR	I	MR	VP	U	CU	Glucose		Lactose		Maltose		Sucrose		Tentatively Identified as:
									A	G	A	G	A	G	A	G	
PCB2	+	+	-	-	-	-	-	+	-	-	+	-	-	-	+	-	PlanococcusSpp

Note: C=Catalase, O=Oxidase, NR= Nitrate Reduction, I= Indole, M.R= Methyl Red, V.P= Voges Proskauer, C.U= Citrate Utilization, U= Urease, A= Acid Production, G= Gas Production, += Positive-= Negative.

The molecular characterization is often advised to further confirm the identity. As a result, molecular characterization was completed using 16S rRNA sequence homology. The isolate was identified by molecular analysis as Planococcus plakortidis strain (figure-06) the isolatehasnot been reported in literature as in chicken gut cecum.

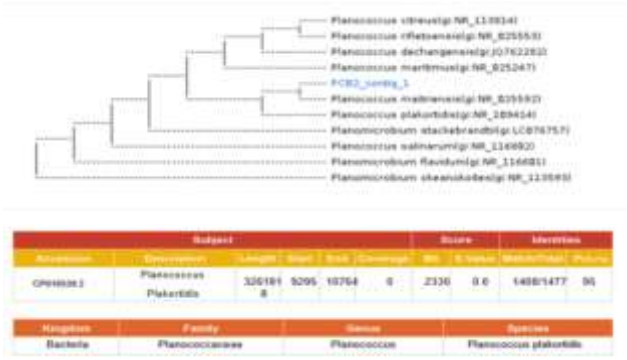

Figure-6: Evolutionary relationship and Similarity index of bacterial strain: PCB2

CONCLUSION

In this study, five bacterial species were screened out from 60 samples based on microscopic examination, cultural characteristics, biochemical test (MR-VP, Oxidase, Catalase, Citrate utilization, and Indole test), and molecular techniques. Results revealed that Planococcusspp, based on biochemical properties. This study also shows the highest growth of all the identified chicken gut bacteria with cellulose on the 10th day, whereas the highest growth with pectin was observed on the eighth day. The phenotypic, genotypic, and phylogenetic relationship analysis showed that strain PCB2 merits recognition as a representative of a novel species of the genus Planococcus, for which the name Planococcus plakortidi is proposed as a novel isolate in this study.

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