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ISOLATION, SCREENING AND MOLECULAR IDENTIFICATION OF AMYLASE PRODUCING BACTERIA ISOLATED FROM DAIRY PRODUCTS

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ABSTRACT: Probiotic bacteria are the favored source of enzyme appropriate to a diversity of industries, such as food, newspaper, cleaner, and energy. The aim of this study was to isolate, molecular characterization and screen probiotic bacteria for amylase production. In this study, isolation, identification, and molecular characterization of probiotic bacteria from different dairy samples were carried out. Later, biotechnologically important enzyme production potentials of the isolates were determined. The results revealed that, the amylase from probiotic bacteria were able to hydrolyze starch into glucose and dextrin's where halos zone around bacteria growth was observed with twelve bacteria plates. The twelve bacteria strains were identified as *Levilactobacillus brevis, Lactiplantibacillus plantarum, Lactococcus lactis* and *Lactiplantibacillus argentoratensis*. This study concluded that, amylolytic activity of beneficial microbial encourage the application of the amylase enzyme in many bio-industrial applications.

Keywords: Probiotic bacteria, Amylase enzyme, Amylolytic activity, 16s rDNA PCR**.**

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1. INTRODUCTION

Probiotics are described as live bacteria that, when given in sufficient concentrations, provide

health advantages to the host. Probiotic bacteria should be able to withstand and survive the harsh circumstances found in the host's intestine. It should arrive at the action site in a physiologically viable condition and defend itself against pathogens by creating antimicrobial compounds such as bacteriocins or metabolites such as organic acids. Lactic acid bacteria are the most common probiotic bacteria that have favorable effects on the GI tract of the host [1]. *Lb. acidophilus, Lb. plantarum, Lb. rhamnosus, Lb. paracasei, Lb. casei,* and *Lb. gasseri are among the Lactobacillus* species. LAB is a group of probiotic bacteria that consists of genus such as *Streptococcus, Lactococcus, Pediococcus, Enterococcus, Lactobacillus* is commonly found in dairy and fermented foods [2]. [3], [4], [5]. There are many studies reporting the health benefits of fermented dairy products. Fermented foods typically contain microorganisms considered to be generally regarded as safe (GRAS), which can produce a range of beneficial by-products / metabolites such as antimicrobial peptides (e.g., bacteriocins), ethanol, organic acids, fatty acids, carbon dioxide [6]. [7]. It is known that products resulting from LAB-induced fermentations have anti-cancer [8]. immunomodulatory [9], anti-gastritis [10], [11], Antihypertensive [12], [13] and anti-allergenic effects [14], [15]. Amylases are the enzymes catalyze the initial hydrolysis of starch into short oligosaccharides through the cleavage of a-D 1, 4 glycosidic bonds [16]. [17]. Starch hydrolyzed with amylase overcomes the acidic nature and helps in maintaining high temperature and there by producing fructose syrup. Various sources like plants, animals, bacteria, fungi, and yeast secrete amylases as an extracellular enzyme. Present day the commercially available amylases are obtained from microbial sources. The advantage of using microbial amylases in industrial application is that they are more stable than any other source and easy to manipulate to obtain enzyme of desirable characteristics in bulk production and economical [18]. [19]. Amylase enzyme is produced by some lactic acid bacteria. *Lactobacillus* that produces amylase is found in the gastrointestinal tracts of chickens, pigs, horses, rabbits, and humans, including babies [20]. [21]. Amylases are gaining popularity due to their potential commercial applications in starch liquefaction, brewing, textile sizing, paper, and detergent sectors. However, amylase's use has expanded to include medical, clinical, and analytical chemistry, where it is used in the formulation of lotions, ointments, and the creation of biopolymers such surgical sutures and controlled drug delivery systems. lactic acid bacteria with amylolytic activity include *Lactobacillus fermentum, Lactobacillus manihotivorans*, and *Lactobacillus fermentum* [22]. Because *Lactobacillus* amylases are non-pathogenic and the end product of fermentation is lactate, which is utilized as a flavoring ingredient in the food industry, their use is regarded safe [23]. Tevea et al. [24] investigated a very thermostable a-amylase enzyme isolated from soil from *Lactobacillus fermentum* 04BBA19. Using *Bacillus amyloliquefaciens 04BBA15, Lactobacillus fermentum 04BBA19,*

and *Saccharomyces cerevisiae*, Fossi et al. [25] investigated microbial interactions for increasing amylase production. *Bacillus amyloliqueaciens 04BBA15, Lactobacillus fermentum 04BBA19* and *S. cerevisiae* was used to boost amylase output. In the manufacturing industry, starch is a cost-effective carbon source. The use of starch and *amyloprobiotic* bacteria together improves the fermentation and produces lactic acid in a single step, lowering the general fermentation cost. The primary goal of this study was to isolate, molecular characterization and screen probiotic bacteria for amylase production.

2. MATERIALS AND METHODS

2.1 Probiotic Bacteria Isolation

Probiotic bacteria were obtained from dairy product samples in Malaysia. appropriate volume of dilution was blended with 90 ml 0.1% peptone water then spread on MRS plates with 0.8 percent calcium carbonate. Plates were incubated in an anaerobic jar to 48 hours at 37°C. Catalase activity was determined for each isolate by introducing a drop of 4% hydrogen peroxide solution to the cells. Bubbles appeared almost instantaneously, showing that catalase was present in the cells. Gram-staining was performed only on catalase-negative isolates. To get pure isolates, the morphology was evaluated using a Nikon microscope and streaked on MRS agar. All of the bacterial species used in this research were stored at -20°C in a 15 percent glycerol stock. Re-culturing was done in MRS broth at anaerobic conditions at 37°C. bacterial strains were sub-cultured no more than four times (1 percent, v/v) at 24-hour intervals prior to the start of the tests (Kheadr, 2006) [26].

2.2 Screening of Amylolytic Activity by Probiotic Bacteria

The Isolates were inoculated into the suitable media to detect amylase activity, and the clearance zone was evaluated. Amylase activity was determined by inoculated bacterial strains into MRS (2% starch, 0.5 % peptone, 0.7% yeast extract, 0.2% NaCl, and 1.5% agar). Following the incubation, clearing zone was observed using Gram's iodine as a detection agent [27].

2.3 Molecular Identification of the eight Strains Probiotic Bacteria Using 16s rDNA PCR

Jarvis & Hoffman (2004) [28]. method was used to molecular identification of the six strains of probiotic bacteria. Bacteria DNA was extracted from an overnight culture in 20 ml MRS broth at 30 ºC using DNA Purification Kit (USA). The pellet was collected after centrifugation of 1ml of overnight bacterial culture11500 rpm for 10 min at 25 ºC (Eppendorf centrifuge). 150 µl of (TE buffer) was added to the pellet then incubation at 37 ºC. 150 µl of gram-positive lysis was mixed with1 µl of proteinase K (Sigma) and then the TE buffered was added to the mixture then thorough mixing. The sample was incubated for 15 min at 65-70 ºC after that vertexing for two min. The sample was placed for 5 min on ice. The following step

was by adding 175 µl of reagent (protein precipitation) to each sample, then centrifugation for 10 min at 4 ºC at 13,000 rpm. The pellets were discarded, and the cell free supernatant was moved to clean tubes. Next step to each sample1 µl of RNase II (5 µg/µl) was added and thorough mixing followed by incubation for 30 min at 37 ºC. Cell free supernatant was mixed with 500 µl of isopropanol followed by centrifuging 10 min at 13,000 rpm. DNA pellet was kept in place. 200 µl (ethanol 70%) was used to washes DNA pellet and centrifuged 2 min at 5, 000 rpm. DNA was resuspended in 35 µl of deionized After remove the ethanol. DNA was stored at -20 ºC for future study.

2.4 Gel Electrophoresis of 16s rDNA PCR Product

Gel electrophoresis analysis was done for the PCR products from universal primer for expected size. 2µl of PCR product mixture was put in electrophoresis for 45 min in 1.5% (w/v) agarose gels in 0.5 x TEA buffer. DNA marker (250 to 10,000 bp) from 1st Base, Malaysia was using as standard. Ethidium bromide was used to stain electrophoresis gels then rinsing the gels. Photographs was taken with UV transilluminator (Bio-Rad, Italy). The six probiotic bacteria primers sequences of16S rDNA were founded by 1st Base, Malaysia. The sequences were compared with the databases (Gen-Bank).

3. RESULTS AND DISCUSSION

Thirty-five of probiotic bacteria that isolated from dairy products were identified as lactic acid bacteria because they produced clear zone on MRS agar supplied with CaCO3, Gram positive and catalase negative as in table [1]and figure [1, 2]. Results of biochemical and morphological tests showed bacterial diversity of dairy products. Study by Afridi et al., [29]. bacterial isolates were identified as *Bacillus* and *Clostridium spp*, according to biochemical and morphological characteristics.

Figure 1: lab isolates producing clear zone on modified mrs-caco$_3$ agar

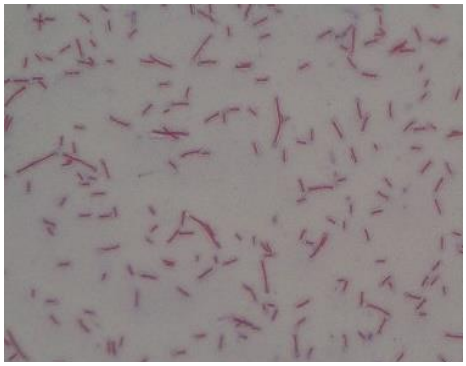

Figure 2: gram positive of lab

3.1 Amylolytic activity of Probiotic bacteria

Microbial products (fungi and bacterial) are the most favorite sources to get amylase enzymes because they easy to cultivate, their short generation, require little space and powerful

purification procedures [30]. Out of thirty-five isolates, fifteen bacteria showed amylolytic activity on starch agar plate. Formation of halos zone around bacteria growth was observed with fifteen bacteria plates, indicating the present of amylolytic activity (starch hydrolysis activity). Amylase's enzyme can used alternative to chemical catalysts and acidic hydrolysis for degradation of starch which is obtainable in large quantities in refuse tank [31]. In the presence of iodine, starch agar plate of other bacteria showed bluish color, due to the absence of amylolytic activity (table [1]and figure [3, 4, 5, 6]. Amylases are important enzyme used in many industries. This enzyme plays important role in starch hydrolysis into glucose and dextrin. Amylase's enzyme used in several industries of food such as pulp and textile [32].

Table 1. Phenotypic characteristics and starch hydrolysis of probiotic bacteria

Sample	Bacterial Codes	Catalase reaction	Gram reaction	Morphology of cell	Starch Hydrolysis
Fermented milk	FM1	-	+	Rod	Negative
	FM2	-	+	Rod	Positive
	FM3	-	+	Short rod	Positive
Yogurt	FM4	-	+	Rod	Positive
	FM5	-	+	Cocci	Positive
	Y1	-	+	Cocci	Positive
Yogurt	Y2	-	+	Short rod	Positive
	Y3	-	+	Short rod	Positive
Soft Cheese	CH1	-	+	Cocci	Positive
	CH2	-	+	Cocci	Negative
	CH3	-	+	Rod	Positive
Cheese Whey	CH4	-	+	Short rod,	Positive
	WH1	-	+	Rod	Negative
	WH2	-	+	Cocci	Positive
	WH3	-	+	Cocci	Positive

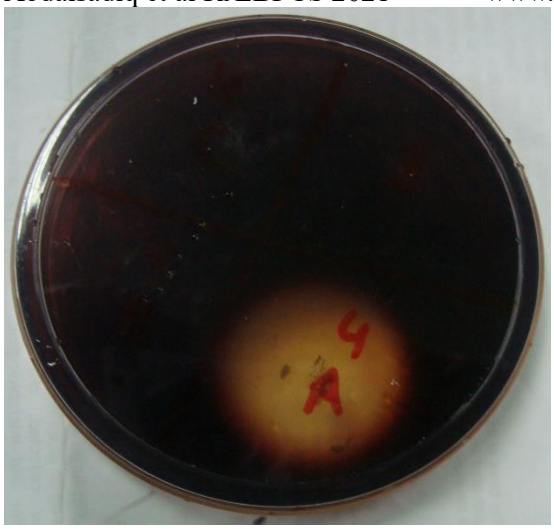

Figure 3: Formation of halo zone by fm4 isolate

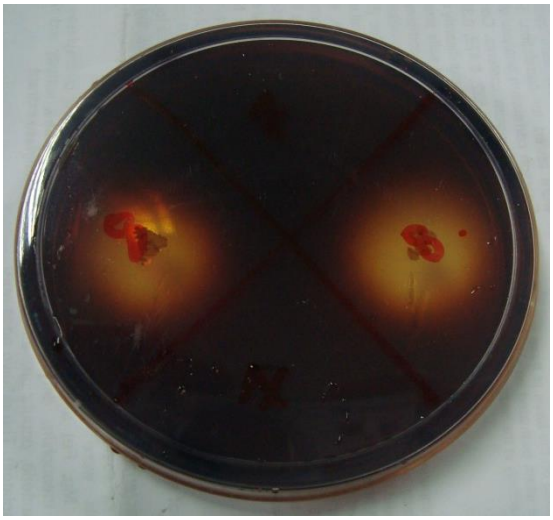

Figure 4: Formation of halo zone by y1 isolate

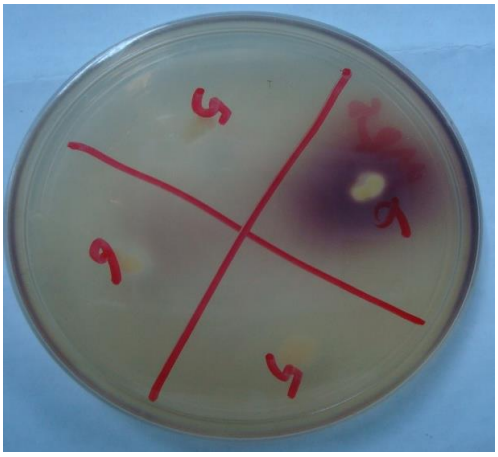

Figure 5: Formation of halo zone by ch3 isolate

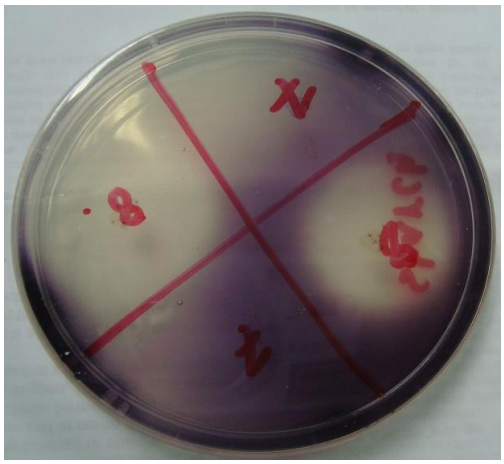

Figure 6: Formation of halo zone by WH3 isolate

3.2 Molecular identification of probiotic bacteria using 16s rDNA with specific primers

Molecular characterization of bacterial DNA using universal primer showed clear strain bands with molecular weight 1400 bp [Figure 7]. The similarity between each strain in this study and other bacteria in data base was estimated as in table 1. The similarity between probiotic isolates (FM1, FM2, FM3, FM4, FM5, Y1, Y2, Y3, CH1, CH2, CH3, CH4, WH1, WH2, WH3) and bacteria from gene bank was estimated by (.100%, 100%, 99.93%, 99.93%, 99.93%, 99.93%, 99.95%, 99.93%, 99.93%, 99.93%, 99.95%, 100%, 99.93%, 99.93%, 99.93%). Different accession number were used to keep each bacterial sequences in the Gene Bank database. The accession numbers of probiotic isolates were (GU2959501, MT61364211, MT6117021, MT6048101, MT6136421, MT5976471, MT5338614, MT5385471, LC3799731, CP0781321,

JQ7236991, JQ4112481, GU1386061, MZ7876271 and OK0364721 respectively [able 2]. This study reported the probiotic bacterial amylase isolated from different dairy products had a molecular weight of1400bp. In previous study, three isolates S17, S5 and S13 were identified by 16S rDNA sequencing of gene and similarity with *Bacillus subtilis, Bacillus tequilensis* and *benzoelyticum* was estimated by 97%as indicated by analysis of phylogenetic [33].

Table 2: Molecular identification of bacterial isolates

Bacterial Codes	Identification	Similarity	Accession No.
FM1	*Levilactobacillus brevis*	100%	GU2959501
FM2	*Levilactobacillus brevis*	100%	MT6136421
FM3	*Lactiplantibacillus plantarum*	99.93%	MT6117021
FM4	*Lactiplantibacillus plantarum*	99.93%	MT6048101
FM5	*Lactiplantibacillus plantarum*	99.93%	MT6136421
Y1	*Lactiplantibacillus plantarum*	99.93%	MT5976471
Y2	*Lactococcus lactis*	99.95%	MT5338614
Y3	*Lactiplantibacillus plantarum*	99.93%	MT5385471
CH1	*Lactiplantibacillus plantarum*	99.93%	LC3799731
CH2	*Levilactobacillus brevis*	99.93%	CP0781321
CH3	*Lactococcus lactis*	99.95%	JQ7236991
CH4	*Levilactobacillus brevis*	100%	JQ4112481
WH1,	*Levilactobacillus brevis*	99.93%	GU1386061
WH2	*Lactiplantibacillus*	99.93%	MZ7876271
WH3	*Lactiplantibacillus plantarum*	99.93%	OK0364721

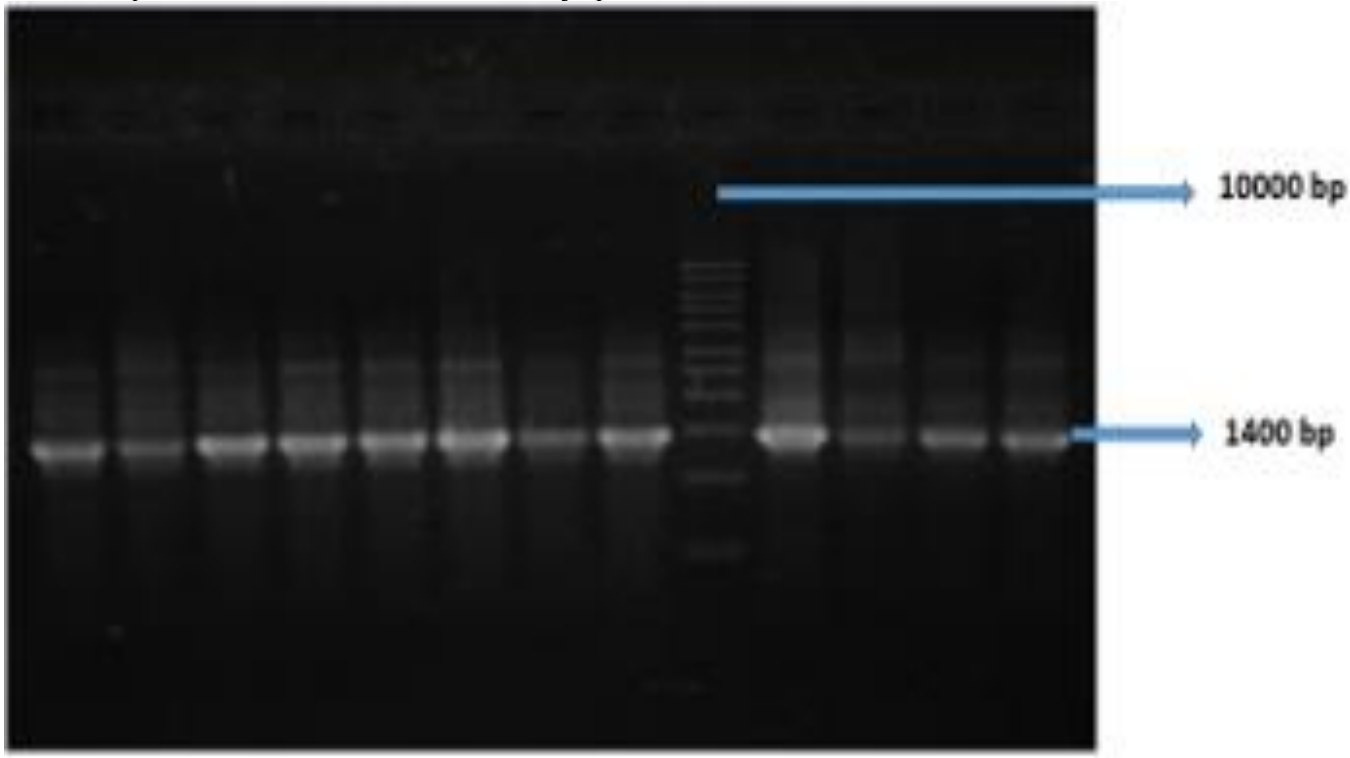

Figure 7: probiotic bacterial DNA bands on the 1.5 % agarose gel using universal primer

4. CONCLUSION

A total of Thirty-five of probiotic bacteria were isolated from dairy products and screened for amylolytic activity. Based on formation of halos zone around bacteria growth, fifteen isolates were capable of starch hydrolysis compared to other probiotic isolates. The fifteen isolates showed high amylolytic activity and identified by PCR, and therefore, these strains of probiotic bacteria. can be considered promising enzyme production.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The author confirms that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

Authors have no conflict of interest.

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