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IDENTIFICATION OF LACTOBACILLUS IN SET YOGHURT SAMPLES AND DETERMINATION OF THEIR ANTIOXIDANT ACTIVITY

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ABSTRACT

Probiotic bacteria Lactobacillus is a non-pathogenic and a live bacterium which is mostly used in the dairy industry to confer health benefits on human hosts when consumed in adequate quantities. The aim of this study was to isolate Lactobacillus from yoghurt and to determine their antioxidant activity. Five yoghurts were cultured on MRS agar and subjected to biochemical tests (Gram staining and catalase test). This was followed by DNA extraction from Lactobacillus using boiled-cell and kit-based method and then quantified using spectrophotometry. Then PCR was carried out to further identify Lactobacillus. Thereafter, antioxidant activity of Lactobacillus was determined by DPPH assay. Creamy white and mucoid colonies having an entire margin with flat, raised, and convex elevations observed on MRS agar, purple rod-shaped bacteria observed during Gram staining, and no oxygen bubbles formed during catalase test confirmed the presence of Lactobacillus. DNA concentration of boiled-cell method was not significantly higher than kit-based method. In DPPH assay the purple solution with DPPH turned light yellow after 30 minutes incubation in dark condition. The scavenging activity of cell intact was slightly higher than cell free. The results obtained from ANOVA test confirmed that There was no significant difference observed in antioxidant activity between cell free and cell intact. In conclusion, the biochemical tests and the PCR confirmed that all five samples contained Lactobacillus and all the bacteria carries antioxidant activity. Our findings could guide the dairy industry to improve and develop new probiotic products and help in treating oxidative induced disease conditions.

Keywords: Probiotics, Yoghurt, Lactobacillus, DPPH, Cell-free, Cell-intact

INTRODUCTION

Probiotic Bacteria

Probiotics are live non-pathogenic bacteria which have beneficial effects on the body when consumed in adequate quantity, by creating a microbial balance in the body. The most common probiotics used are Lactobacillus and Bifidobacterium, this research is focused mainly on the probiotic lactobacillus. The main sources of Lactobacillus include yoghurt, curd, cheese, and fermented beverages (Somashekaraiah et al., 2019; Vanniyasingam, Kapilan and Vasantharuba, 2018). The common probiotic used in diary industry is lactobacillus. Probiotics have many health benefits including boosting the immune system, fighting allergies, improving mental health, and maintaining good digestion (Arshad et al., 2018; Shi et al., 2016).

Lactobacillus is the foremost member of the family Lactobacillaceae. Lactobacillus is a rod-shaped, microaerophilic or a facultative anaerobic bacterium which shows a gram-positive

and a catalase-negative result (Karami et al., 2017; Goldstein, Tyrrell and Citron, 2015). Lactobacillus is a non-spore-forming bacterium which secretes various bacteriocins such as lactacin and acidocin which can improve lactose utilization, control intestinal infection, provide resistance against gastric acid and bile and has antimicrobial properties against harmful microbes (Masalam et al., 2018; Azat et al., 2016; Goldstein, Tyrrell and Citron, 2015). For Lactobacillus to impart their benefits they should survive the acidic gastric environment and reach the small intestine. Lactobacillus rhamnosus GG can be resistant to pH values as low as 2.5 for 4 hours (Corcoran et al., 2005).

Lactobacillus appears on MRS agar as creamy white and shiny white colonies having an entire margin with flat, raised, and convex elevation (Kumar and Kumar, 2014; Forouhandeh et al., 2010). They are normally found in the gastrointestinal tract, urinary and genital system without causing any infection. Lactobacillus is used as a probiotic since it is associated with many health benefits such as antioxidant activity, acid tolerance, cholesterol removal, boosting the immune system, preventing inflammatory bowel syndrome and vaginal infection (Marhamatizadeh and Sayyadi, 2019; Arshad et al., 2018; Karami et al., 2017).

According to Popovic et al., 2020, Ng et al., 2011, Soomro and Masud, 2008 and Jumah, Abu-Jdayil and Shaker, 2001 the starter culture of the yoghurt contains milk and bacteria such as Streptococcus thermophilus and Lactobacillus delbrueckii sp. Bulgaricus which are live, viable and abundant. This Lactobacillus present in the milk converts the lactose to lactic acid, which thickens the milk and creates a distinctive tart flavour and texture to the yoghurt (Bintsis, 2018; Forouhandeh et al., 2010). Lactobacillus also produces acetic acid, ethanol, aroma compounds, exopolysaccharides and bacteriocin which can act as a natural preservative and increase the shelf-life of yoghurt by suppressing the growth of pathogenic bacteria (Forouhandeh et al., 2010; Djenane et al., 2005). In addition to probiotics, yoghurt also contains protein, calcium, riboflavin, phosphorus, iodine, and vitamin B12 (Moore, Horti and Feilding, 2018)

Antioxidant's role in preventing oxidative stress

Oxidative stress is the elevated level of reactive oxygen species, including the superoxide radical, hydrogen peroxide, and hydroxyl radical, that are known to cause oxidative damage including tissues and organs damage (Bierzuńska and Cais-Sokolińska, 2018; Lobo et al., 2010). The antioxidant is used to prevents or delays free-radical induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition (Young and Woodside, 2001).

Lactobacillus is a natural antioxidant that prevents oxidative stress. Lactobacillus exert antioxidative effects through various action. According to a study carried out by Chooruk, Piwat and Teanpaisan, 2017, Lactobacilli can produce different antioxidants including glutathione, glutathione reductase and superoxide dismutase which will reduce free radicals' formation, thereby reducing the oxidative stress. Lactobacillus acidophillus and Lactobacillus bulgaricus acts as metal ion chelating agent by capturing ions like Cu2+ and Fe2+ and preventing them from catalysing the oxidation as stated in Lin et al., 1999.

The Nrf2/Keap1, protein kinase C and Mitogen-activated protein kinase signalling pathway plays a vital role in the antioxidant mechanisms of Lactobacillus to protect the host against oxidative stress. The mechanism of these pathways regulates microbial balance in intestine by lowering the intestinal pH, this prevents the release of endotoxins to the blood and

thereby reduces oxidative stress (Pourrajab et al., 2020; Azad et al., 2018).

There is a high use of artificial antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene to reduce the oxidative stress, but the question is whether these are safe to consume as it can cause damage to the human body leading to liver damage and carcinogenicity (Wu, Cheng and Dong, 2020; Poljsak, Šuput and Milisav, 2013). For this reason, it becomes vital to research more on natural antioxidant which causes less or no side effects. In recent years studies have found out that when probiotic, Lactobacillus are consumed in adequate it enhances the antioxidant level in the body to neutralize the oxidative stress (Wang et al., 2017; Lin and Yen, 1999). The significance of this study was to compare and evaluate the cell-free and cell intact antioxidant activity of Lactobacillus and to compare and evaluate the DNA extraction by boiled cell and kit-based method

This study aims to isolate Lactobacillus from five yoghurt samples and to evaluate the antioxidant activity of yoghurt which confers health benefits to the host by reducing oxygen free radicals in the body. The purpose of this study identifies lactobacillus from yoghurt samples and determine their antioxidant activity. Therefore, the finding of this study will provide useful information to the dairy industry to improve and develop new probiotic products and help in treating oxidative induced disease conditions.

METHODOLOGY

Sample Preparation and Isolation of Lactobacillus bacteria

Five yoghurt samples were purchased from the local market and 5g of each yoghurt sample was homogenized labelled as A to E respectively. MRS agar was prepared and poured into petri plates and allowed to solidify. A loop full of samples was streaked on MRS agar using the quadrant streaking method. The agar plates were incubated at 370C for 48 hours.

Biochemical Characterization of presumptive Lactobacillus

A presumptive Lactobacillus colony was selected, and a smear was prepared on a clean glass slide. Thereafter, gram staining was carried out and purple rod-shaped bacteria were observed.

This was followed by catalase test where presumptive Lactobacillus colony was treated with 3% of H2O2 and evolution of O2 bubbles were observed.

Sub culturing

Pure Lactobacillus colonies identified through biochemical tests were sub-cultured in MRS broth. The subcultures were incubated at 370C for 24 hours.

DNA Extraction

DNA extraction was carried out for all the sample and the positive control using Qiagen DNeasy Blood and Tissue kit and boiled cell method. The extracted DNA was stored at 40C.

Quantification of DNA

DNA absorbance was measured for each sample at 260nm.

Following equation was used to calculate the DNA concentration (1) (Abdulamir et al., 2010).

$$\frac{A260 \times 50\mu g/ml \times DF}{1000}$$

Identification of Lactobacillus by Polymerase Chain Reaction (PCR)

The isolated DNA from Lactobacillus using boiled cell and kit-based DNA extraction method was subjected to genus-specific PCR. Initially, the PCR mixture was prepared with 5µl of PCR buffer, 1.5µl of dNTPs, 2.5µl of forward and

reverse primers, 0.25µl of taq polymerase and 10.75µl of distilled water. From this mixture, 24µl was aliquot into 7 PCR tubes, and 1.0µl of DNA template was transferred into its corresponding tubes, and 1.0µl of autoclaved distilled water was added to the negative control.

The PCR was carried out according to cyclic conditions shown in Table 3.1. Table 3.2 shows the primer sequences that were used in this PCR reaction.

Table 3.1: thermal cyclic conditions of PCR reaction (Modified from: Byun et al., 2004).

Steps	Temperature / ℃	Duration/ minutes	Number of cycles
Initial denaturation	94℃	5	
denaturation	94℃	1	┐
annealing	62℃	1	35
extension	72℃	2	┘
Final extension	72℃	12	
Final hold	4℃	∞	

Table 3.2: primer sequences for the lactobacillus gene (Byun et al., 2004).

Primers	Primer sequences (5'- 3')	Amplicon size (bp)
Forward	TGGAAACAGRTGCTAATACCG	233
Reverse	GTCCATTGTGGAAGATTCCC	233

Visualization of PCR products using agarose gel electrophoresis

1% of Agarose gel was prepared to analyse the PCR amplicons by electrophoresis.

DPPH free radical scavenging assay:

i. Cell Intact

To prepare cell intact, Lactobacillus bacteria was sub-cultured on MRS broth and incubated at 370C for 24 hours. This was centrifuged to obtain a pellet which was resuspended in distilled water and was used as the sample (Azat et al., 2016).

ii. Cell Free

To prepare cell free, 5ml of Lactobacillus overnight subculture was centrifuged at 4000rpm for 15 minutes and boiled for 20 minutes. Then freeze at -20℃ for 20 minutes and centrifuged at 4000rpm for 10 minutes. The supernatant was used as the sample.

This sample obtained from cell intact and cell free was used for the DPPH assay. 1ml of sample and 2ml of DPPH was added in a test tube, for control, 1ml of autoclaved distilled water and 2ml of DPPH solution was added in test tube and for blank, 1ml of sample and 2ml of ethanol was added in test tube. This was incubated for 20 minutes in dark, and absorbance was taken at 517nm. Using the obtained the mean absorbance value, DPPH scavenging activity was calculated by the following equation (2) (Azat et al., 2016).

SA DPPH = [1- (A sample – A blank) / A control] x 100%

Statistical Analysis

DNA concentration and antioxidant activity of cell intact and cell free were statistically analysed using one way ANOVA on SPSS software. P value < 0.05 was considered as statistically significant.

RESULTS

Colony Morphology

Yoghurt samples were streaked on MRS agar to obtained Lactobacillus colonies

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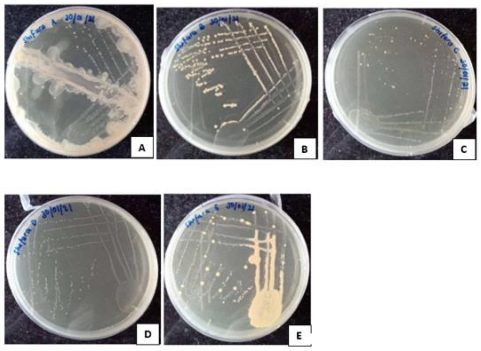

Figure 4.1: Colonies obtained from the first streaking of yoghurt samples in MRS agar

Creamy white colonies having an entire margin with flat, raised and convex elevation was present on MRS agar.

Gram Staining

The colonies obtained from MRS agar were subjected to Gram's staining.

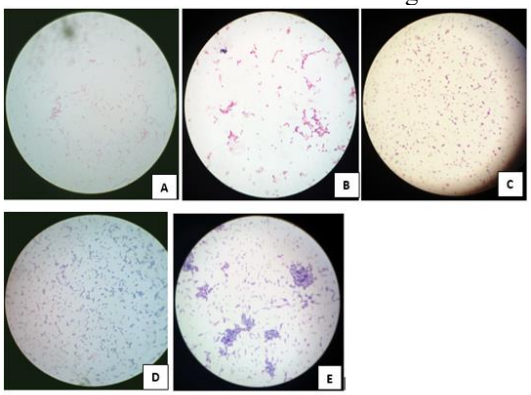

Figure 4.2: *Gram Staining of sample A, B, C, D and E under 100X*

Catalase Test

The colonies obtained from MRS agar were subjected to catalase test to detect evolution of gas bubbles.

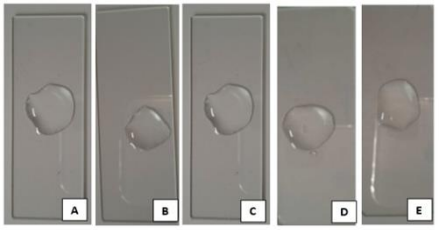

Figure 4.3: *Catalase test for yoghurt samples.*

Evolution of oxygen bubbles were not observed in all 5 samples A, B, C, D, and E confirming that the bacteria present is catalase negative.

DNA concentrations of boiled cell and kit-based methods

Table 4.1: DNA concentration obtained from boiled cell and kit-based method

Samples	Boiled cell method (ng/ml)	Kit-based method (ng/ml)
A	174.7 ± 0.000577	225.0 ± 0.001155
B	605.2 ± 0.000707	540.0 ± 0.001155
C	172 ± 0.000577	345.0 ± 0.000577
D	123.8 ± 0.000577	427.5 ± 0.001155
E	150.0 ± 0.011547	412.5 ± 0.000577

The DNA concentration obtained from boiled method was higher compared to kit-based method.

Table 4.2: ANOVA table for DNA Concentration

ANOVA

Concentrations

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.672	1	7.672	2.978	.123
Within Groups	20.608	8	2.576		
Total	28.280	9			

The obtained p value was greater than 0.05 which means there is no significant difference between the DNA concentration of the boiled cell method and kit-based method.

Visualization of agarose gel electrophoresis

The PCR products obtained from boiled call and kit-based method were visualized using agarose gel electrophoresis

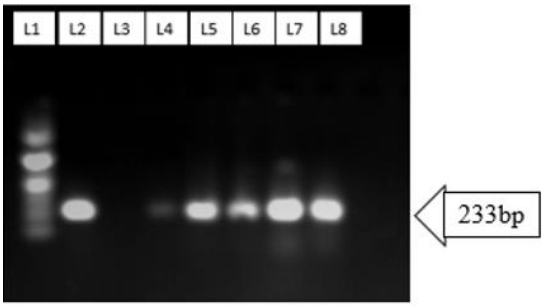

Figure 4.4: Gel image of PCR products of boiled cell method (L1- 100bp DNA ladder, L2- positive control, L3- negative control, L4- sample 1, L5- sample 2, L6- sample 3, L7- sample 4, L8- sample 5).

Bands were observed at 233bp for all five samples along with the positive control and no band was observed for negative control.

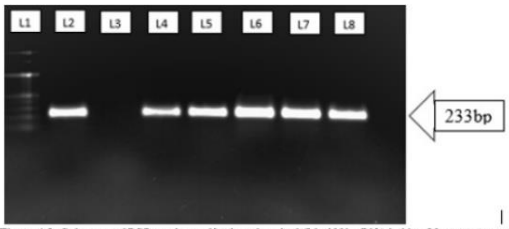

Figure 4.5: Gel image of PCR products of kit-based method (L1- 100bp DNA ladder, L2- positive control, L3- negative control, L4- sample 1, L5- sample 2, L6- sample 3, L7- sample 4, L8- sample 5).

Bands were observed at 233bp for all five samples along with the positive control and no band was observed for negative control.

DPPH Free Radical Scavenging Assay for Cell free sample

The cell free and cell-intact samples was assessed using DPPH assay to determine the scavenging activity.

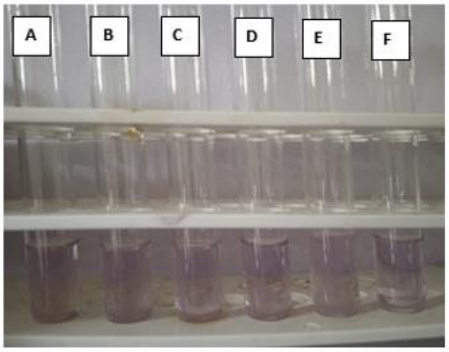

Figure 4.6: Colour change before incubation in dark

The solution was light purple in colour before incubation in dark.

Figure 4.7: Colour change after 30 minutes incubation in dark

The solution turned light yellow after incubation in dark.

DPPH Free Radical Scavenging Assay for Cell intact sample

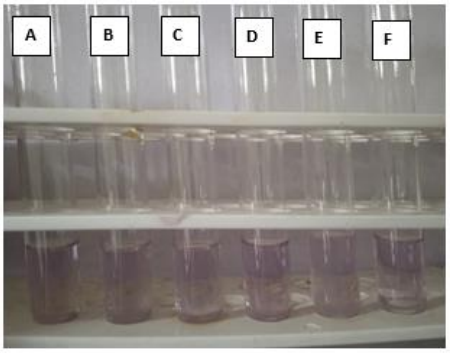

Figure 4.8: Colour change before incubation in dark

The solution was light purple in colour before incubation in dark.

Figure 4.9: Colour change after 30 minutes incubation in dark

The solution turned light yellow after incubation in dark.

Scavenging activity values of cell free and cell intact

Table 4.3 depicts all the scavenging activity values of cell free and cell intact with their respective standard deviation.

Table 4.3: Antioxidant activity of cell free and cell intact

Sample	Scavenging Activity of cell free (%)	Scavenging Activity of cell intact (%)
A	62.95 ± 0.353553	71.95 ± 2.05061
B	50.75 ± 0.777817	73.05 ± 0.494975
C	74.85 ± 1.06066	34.2 ± 1.555635
D	64.5 ± 1.838478	41.25 ± 1.626346
E	37.05 ± 1.767767	83.8 ± 1.555635

The scavenging activity of cell intact was found to slightly higher than cell free sample.

Table 4.4: ANOVA table for antioxidant activity

ANOVA
Antioxidant

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	40.045	1	40.045	.131	.721
Within Groups	5490.841	18	305.047		
Total	5530.886	19			

The obtained p value was greater than 0.05 which means there is no significant difference between the scavenging activity of cell-intact and cell free sample.

DISCUSSION

In this study, Lactobacillus bacteria was identified in yoghurt samples and were investigated for their antioxidant activity. According to Goldstein et al., 2015 the colony morphological features of Lactobacillus were confirmed to be creamy, shinny, and mucoid colonies having an entire margin with flat and raised elevations. This morphology was observed in this study on colonies formed in the MRS agar as shown in figure 4.1. Based on this evidence it can be deduced that the colonies formed on MRS agar may be due to lactobacillus. (Nachi et al., 2019).

For confirming the presence of Lactobacillus in yoghurt samples

biochemical tests including Gram staining and catalase test were carried out. According to study carried out by Ismail et al., 2018, it confirms that Lactobacillus is a purple rod-shaped gram-positive bacterium. As also figure 4.2 for Gram staining shows purple rod-shaped bacteria in all five samples under 100X. Therefore, it confirms that samples may contain Lactobacillus.

According to Iwase et al., 2013 in catalase test, hydrogen peroxide (H2O2) is hydrolysed into oxygen and water in presence of enzyme catalase. when bacterial isolates do not produce catalase evolution of O2 bubbles will not be observed. Figure 4.3 shows that no oxygen bubbles were evolved from all five samples when H2O2 was added. This proves that the samples may contain Lactobacillus. The results obtained for the biochemical results were similar to the previous studies confirming the presence of Lactobacillus in the sample and that Lactobacillus is a gram positive, and a catalase negative bacterium.

From the isolated Lactobacillus, DNA was extracted using boiled cell method and kit-based method. These DNA was quantified using spectrophotometry at 260nm. The mean DNA concentration of boiled cell is 214.2 ng/µl and kit based is 390 ng/µl. According to the results provided on the table 4.1 it shows that the DNA extracted by boiled cell method were in higher concentration than the kit base method. However, this difference was not statistically significant ($p > 0.05$) as shown in table 4.2. Similar results were found by Oliveira et al., 2014 which helps to confirm that boiled cell method produce higher DNA concentration compared to kit-based method (Junior et al., 2016; Yamagishi et al., 2016).

According to Ahmad and Dablool, 2017, the DNA extraction by boiled cell method could be advantageous over many commercial kits, because it is of low cost, fast, safe and can be applied for high-yield isolation of analytical quality DNA. In addition, it can be used in laboratories lacking supplies, equipment, and technology.

For further confirmation of Lactobacillus, Polymerase chain reaction (PCR) was carried out. The PCR products obtained were 233bp in length which confirms that the samples contained Lactobacillus. According to a study carried out by Byun et al., 2004 using the genus-specific primers, amplicon size of Lactobacillus was found out to be 233bp. Figure 4.4 is the PCR products of boiled cell extraction method which shows thick bands at 233bp for all 5 samples and figure 4.5 is the PCR products of kit-based extraction which also shows bands at 233pb for all 5 samples confirming the presences of Lactobacillus.

The antioxidant activity of both cell free and cell intact were checked by the DPPH free radical scavenging assay. DPPH is most used to evaluate the antioxidant activity as it is a stable compound that can be reduced by accepting hydrogen or electrons showing colour change (Salari et al., 2019). The purple DPPH solution had changed to yellow indicating that there is a scavenging activity of cell free and cell intact against DPPH free radicals. The figure 4.6, 4.7, 4.8 and 4.9 shows the colour change observed before and after 30 minutes incubation under dark condition. The degree of discoloration of DPPH solution indicates the scavenging potential of the antioxidant compounds.

The DPPH solution is light sensitive which turns the solution from purple to yellow in presence of light. Therefore, it is necessary to incubate the samples in dark condition to maintain the accuracy of the result. Scavenging activity of cell intact sample was slightly higher than cell free sample. This may be because some lactobacillus species may have better antioxidant activity than others (Chooruk, Piwat and Teanpaisan, 2017). Scavenging activity of both cell free and cell intact

sample was found to be high. According to the statistical analysis performed using SPSS software, One-way ANOVA test table 4.6 depicts P value as 0.068. A P >0.05 was considered as statistically not significant. Therefore, there is no statistically significant difference between antioxidant activity of cell free and cell intact as the P value is greater than 0.05 (Mu et al., 2018 and Xing et al., 2015).

The antioxidant activity of cell free may be due to proteins and exopolysaccharides and the antioxidant property of cell intact is due to the presence of casein, amino acids, vitamins (A, C, D and E), β carotene and enzymes. (Yang et al., 2020; Fardet and Rock, 2017; Xing et al., 2015).

CONCLUSION

In conclusion, according to the results obtained by biochemical tests and PCR results confirmed that all samples contained Lactobacillus. Both cell free and cell intact samples contained antioxidant activity and confirmed that there was no significant difference between scavenging activity of both cell free and cell intact sample.

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