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Isolation of Lactobacillus and Enzymatic Activity of Dihydrofolate Reductase (DHFR) in Musa spp.

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ABSTRACT

Background: Bananas cultivated in tropical and subtropical regions rank as crucial global crops. Banana is a general term embracing a number of species or hybrids in the genus Musa, family *Musceae*. ***Objective:*** The current study thoroughly examines the advancements in harnessing the potential of Musa, i.e., bananas, throughout their transition from unripe to ripe stages, with the aim of determining the presence of Lactobacilli and evaluating the enzymatic activity of Dihydrofolate Reductase (DHFR). DHFR, a ubiquitous enzyme, is involved in the metabolism and stability of folate (vitamin B9), and causes the reduction of dihydrofolate into tetrahydrofolate (folate or vit B9), which is utilized in amino acids and purine production. It has been studied that the deficiency of DHFR or its inhibition can lead to Megaloblastic anemia, Neural tube defects, improper synthesis of DNA and RNA, colorectal cancer, etc. The presence of the DHFR enzyme in bananas can be utilized in the treatment of diseases related to deficiency of folate, which can become a cheaper and cost-effective way to keep a balance in the folate metabolism and keep the activity of the enzyme maximum. ***Methodology:*** The fresh unripe banana samples were collected from an urban fruit market in Karachi, serially diluted, and inoculated into the Nutrient agar, Mannitol salt agar, and De Man, Rogosa, Sharpe agar media to check for microbial growth. The enzymatic activity of DHFR and its association with Musa was also checked by using DHFR substrate. ***Results:*** The results concluded from our experiment are that the Musa contains Gram-positive bacilli that were further tested to characterize the microorganism into its respective genus, indicating the presence of Lactobacillus in the banana. The enzymatic activity of DHFR was observed at maximum on day 2 and minimum enzymatic activity was observed on day 4. ***Conclusion:*** Hence, people suffering from folic acid deficiency, DHFR enzyme and consumption of day 2 bananas can be prescribed. Not only this, the presence of Lactobacilli as probiotics in bananas restore consumer's intestinal microbial dysbiosis and maintain healthy intestinal ecosystem. Furthermore, herbal medicines can be made from banana and given to patients in order to restore the deficiency.

KEYWORDS: Musa, Dihydrofolate Reductase, Megaloblastic Anemia, Neural Tube Defects.

1. INTRODUCTION

Edible plants, including bananas, play a very significant role in ecosystem development and biodiversity. The exudates obtained from various plant sources and their respective components hold significant promise as food additives due to their antimicrobial, antioxidant, and health-enhancing properties (Nikmaram N *et al.*, 2018). Further, plants belonging to different families have been extensively used to treat infections, and diseases in human history (Anne 2007).

The banana, a tropical fruit classified within the Musaceae family, is cultivated in numerous countries across the globe (Shadma A *et al.*, 2014). There are more than 70 different species of Musa with a broad variety of applications. The most common is banana scientifically known as *Musa sapientum* (Fereidoon et al., 2004), which is used widely because of its nutritive value. Various components of the banana plant, such as its flower, pulp, stem, and leaves, have been employed for medicinal purposes in the treatment of anemia, depression, heartburn, strokes, stress, etc. (Imam MZ et al., 2011; Roy A and Saraf S, 2006; Girish and Satish S, 2008; Mokbel MS and Hashinaga F, 2005).

While the banana peel is robust, it can serve as a habitat for indigenous microorganisms, including Lactic Acid Bacteria, which can gain access through skin penetration, natural openings, or mechanical damage (Oyewole OA, 2012). Lactobacilli are characterized as Gram-positive, non-spore-forming rods that are catalase-negative (Bernardeau et al., 2008). These LAB, when used as probiotics, offer benefits in managing gastrointestinal conditions, such as diarrhea and antibiotic-induced imbalances (Sullivan and Nord, 2005). Numerous studies have documented the isolation of Lactic Acid Bacteria (LAB) from a variety of fruits, vegetables, and their byproducts,

as reported by various researchers (Zlatica Kohajdova et al., 2006). Mayer and Hillebrandt (1997) conducted research on the isolation and characterization of LAB strains derived from potatoes, encompassing species like L. brevis, L. casei, L. delbrueckii, L. helveticus, L. lactis, and L. plantarum. Moreover, El-Rahim and colleagues (2017) isolated LAB strains based on their physiological and biochemical characteristics, which have demonstrated the ability to mitigate the pathogenicity of infections, not only within the intestines but also in extraintestinal sites (Peral et al., 2009), including the stomach (Park et al., 2007). LAB produces organic acids, which can contribute to a reduction in intestinal pH and inhibit the growth of other bacterial pathogens (Fang et al., 1996).

Dihydrofolate Reductase (DHFR) is an enzyme essential for folate metabolism and DNA synthesis, playing a vital role in cellular processes (Goodey NM et al., 2011; Goodey NM and Benkovic SJ, 2008). This NADPH-dependent enzyme catalyzes the conversion of dihydrofolate to tetrahydrofolate. Inhibiting DHFR activity halts DNA synthesis, leading to cell death. Consequently, DHFR has become a prominent target in medicinal chemistry for the development of anticancer, antibacterial, and antimalarial drugs (Lamb KM et al., 2013).

The present research delves into an extensive exploration of using Musa, specifically bananas, throughout their transition from unripe to ripe stages, with the aim of assessing the presence of Lactobacilli and evaluating the enzymatic activity of Dihydrofolate Reductase.

2. MATERIALS AND METHOD

2.1 Sample Preparation

The fresh unripe (green) banana samples were collected from an urban fruit market in Karachi. Half of the unripe, day 1 banana was soaked into 10 ml saline; it was then stirred for 10 minutes until most of the puree was mashed, homogenized, and transferred into a beaker and kept separately for preparation of serial dilution.

2.2 Identification of Microbial Isolates:

From day 1-4 bananas were removed from the incubator, serial diluted and inoculated into the Nutrient agar, Mannitol salt agar, and De Man, Rogosa, Sharpe (MRS) agar media plates and again kept in the incubator for 24 hours for the isolation and identification of microorganisms. All bacteriological media were purchased from Oxoid (UK). Microbial isolates were identified by using Gram reaction, catalase, motility, oxidase, and IMVC tests.

2.3 Antibiotic susceptibility Assay

A disc diffusion assay was conducted to assess the antibiotic susceptibility of the isolated strains from banana samples. Antibiotics ciprofloxacin, gentamicin, erythromycin, and ampicillin were purchased from Oxoid (UK).

2.4 Enzymatic Activity of DHFR

2.4.1 Cell Lysis

For an enzymatic activity, the remaining half of the banana was mashed using a mortar pestle until it became puree. The first step was the preparation of a salt-detergent solution for which 80 mL distilled water, 10 mL liquid detergent, and 10 grams non-iodized salt were weighed and mixed uniformly on a magnetic stirrer and added into the mashed banana puree. The mixture was then strained. In the meantime, the meat tenderizer solution was made by mixing 45 mL of distilled water with 5 grams of meat tenderizer powder and then was added to the sample solution. Followed by the addition of cold ethanol to the sample solution until the bubbling stopped.

2.4.2 Enzymatic Activity

Before checking the activity of DHFR, some solutions were prepared.

i. DHFR (substrate)

In a 10 ml beaker, substrate along with (2200 µl) Assay buffer 10x was emptied and stirred for 60 seconds during which its pH was maintained at 7.50 by adding Hydrochloric acid.

ii. 10 mM NADPH stock solution

To prepare this solution, 200 µl of Assay buffer10x, 9.8 ml distilled water was added into the NADPH bottle (provided with the kit), shaken uniformly and stored at -20^{o}C.

iii. Assay Buffer 1x

To prepare this buffer, 5.0 ml of Assay buffer 10x was diluted to Assay Buffer 1x by using 45 ml distilled water and then stored at room temperature. The mentioned solutions were used in different ratios as we tested the presence of DHFR enzyme in the banana filtrate.

Blank 1 was prepared in an Eppendorf therefore 990 µl of the Assay buffer 1x, 10 µl DHFR enzymes, and 6 µl NADPH were mixed together; in the meantime, Blank 2 was prepared using 990 µl Assay Buffer 1x, 10 µl DHFR enzyme (along with the kit) and 5 µl DHFR substrate. Next Eppendorf's prepared were Reaction 1 and 2, both of which contained 990µl Assay Buffer 1x, 6µl of NADPH, 5 µl of the DHFR substrate however in Reaction 1,10 µl DHFR enzyme; while in Reaction 2, 10 µl sample cell extract was added.

3. RESULTS & DISCUSSION

Folate deficiency is the major cause of nutritional anemia in Pakistan which affects approximately 83% of the population out of which it is affecting around 64% of females that undergoes multiple pregnancies and 36% males. Folate is prescribed to women before conception in order to minimize birth defects including Neural Tube defects such as Spina bifida and anencephaly. Our results depict the enzymatic activity of DHFR from the banana lysate **(**Figure 1 and Table 4**)**. From the current study, it has been observed that day 2 banana, yellow-greenish in color contains the maximum enzymatic activity of Dihydrofolate Reductase, whereas, day 4 showed minimal enzymatic activity. DHFR contributes as a fundamental enzyme in various enzymatic reactions including the reduction of Dihydrofolate to Tetrahydrofolate while using NADPH as an electron donor. During the study, the ripening stage of the banana was also observed i.e. when the unripe banana was kept in a cool environment the ripening stage was slowed down whereas when it was kept in a warm place the ripening stage was greatly enhanced. The lysates produced from Day 1 – 4 bananas were translucent in solution with black threads (DNA) settled on the surface. The antibacterial activity of plants can be attributed to the presence of enzymes and various secondary metabolites, including flavonoids, tannins, alkaloids, and terpenoids. (Goodey *et al.*, 2011; 2008).

Tables 1: Colonial Morphology of isolated bacteria from banana samples

Days	**Color**	**Type of Colony**	**Microscopic Morphology**	**Staining Properties**	
			Cell form and arrangement	*Gram Staining*	*Endospore Test*
Day-1	Dark green	Pin pointed	Rod	+	-
Day-2	Light green	Large pin pointed	Cocco bacilli	+	-
Day-3	Light greenish yellow	Small pin pointed	Cocci in pairs	+	+
Day -4	Yellow with a black patch	Pin pointed	Cocci in long chain	+	-

Table 2: Biochemical Analysis of bacterial isolates from banana samples.

Days	**Motility Test**	**Catalase Test**	**Indole Test**	MR **Test**	**Vp Test**	**Citrate Test**	**Oxidase Test**
Day 1	Non-motile	-	-	-	-	-	-
Day 2	Non-motile	-	-	-	-	-	-
Day 3	Motile, non-motile	+	+	+	-	+	+
Day 4	Non motile	-	-	-	-	-	-

Table 3: Antibiotic Susceptibility of Lactic acid bacteria isolated from banana samples.

Isolates	Zone of Inhibition (in mm)			
Lactic acid bacteria	Ciprofloxacin (5ug)	Streptomycin (10ug)	Ampicillin (10ug)	Erythromycin (10ug)
	-	-	-	-

- **= No zone of Inhibition**

Table 4: Enzymatic analysis of DHFR

Sample	Standard	Enzyme
Blank	**0**	**0**
Day 1	0.041	0.014
Day 2	0.004	0.005
Day 3	0.002	0.001
Day 4	0.005	0.007

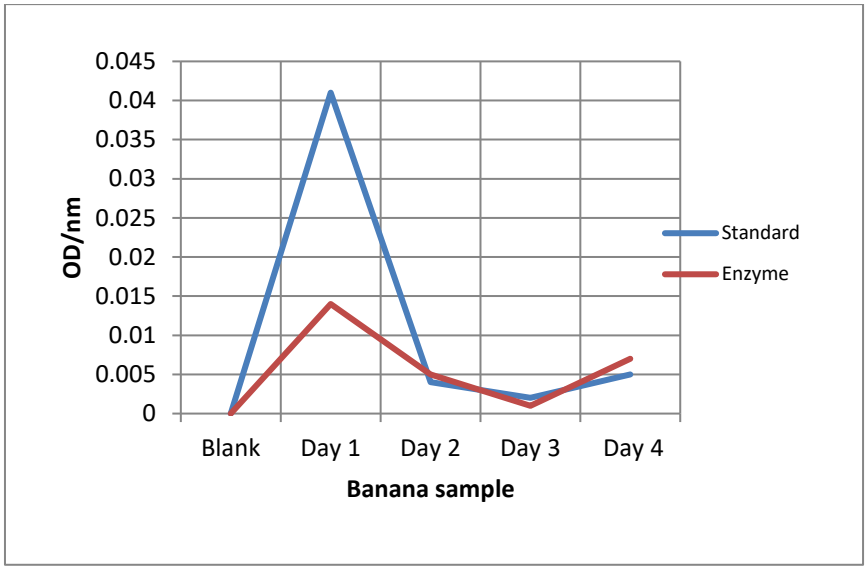

Figure 1: Enzymatic activity of DHFR from the banana lysate
(Blue line indicates the standard, whereas the red line indicates the activity of DHFR enzyme in units/mg P from day 1 to day 4 Musa)

Furthermore, the purees prepared from Day 1 and 4 bananas indicated the presence of Gram-positive short-chain cocci, while in Day 3 banana puree, both Gram-negative and Gram-positive bacteria were observed. The biochemical characterization of the banana indicated the presence of the Lactobacillus bacteria as the isolates were mostly Gram-positive, catalase-negative, non-motile, non-endospore forming and IMVIC tests negative. Lactobacilli are considered as GRAS and can be used as probiotics with many beneficial roles in human health. Hence the presence of Lactobacilli as probiotics in banana restore intestinal microbial dysbiosis and maintain healthy intestinal ecosystem. Tables 1 and 2 exhibit morphological characteristics, IMVC, catalase, oxidase, endospore, and motility tests for the identification of isolated bacteria from banana samples. In the study, the Gram-positive microorganisms which were found in the banana were Bacillus serfenis, Lactobacillus acidophilus, Lactobacillus casei, Cellulomonas; the Gram-negative bacteria found were Enterobacter and Pseudomonas.

In addition, our banana samples LAB isolates were resistant to all tested antibiotics as shown in Table 3 and Figure 2. The presence of Lactobacilli as probiotics in banana restores consumers' intestinal microbial dysbiosis and maintains a healthy gastrointestinal ecosystem. These beneficial microbes reduce the severity of infections and have a positive impact on intestinal and extra-intestinal sites (Peral *et al.*, 2009); (Weizman *et al.*, 2005).

4. CONCLUSION

From the current study, it has been observed that day 2 banana, yellow-greenish in color contains the maximum enzymatic activity of Dihydrofolate Reductase, whereas, day 4 showed minimal enzymatic activity. The presence of Lactobacilli as probiotics in banana restore consumers' intestinal microbial dysbiosis and maintain a healthy intestinal ecosystem. Hence, people suffering from folic acid deficiency, DHFR enzyme, and consumption of day 2 bananas can be prescribed. Furthermore, herbal medicines can be made from bananas or edible vaccines can be prepared and given to patients in order to restore the deficiency.

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