Microbial synthesis of eco-friendly polylactate plastic from low-cost agro-industrial wastes as an alternative to petrochemical-based plastic

Anwar M.[1], Hassan N.[1], Maii E. Youssef[1], Galal A.[2], Elhariry H.[3], Lotfy M.[4*], Abou-Taleb Kh.[4*]

[1] Biotechnology Program, Faculty of Agriculture, Ain Shams University, Hadayek Shubra, Cairo 11241, Egypt

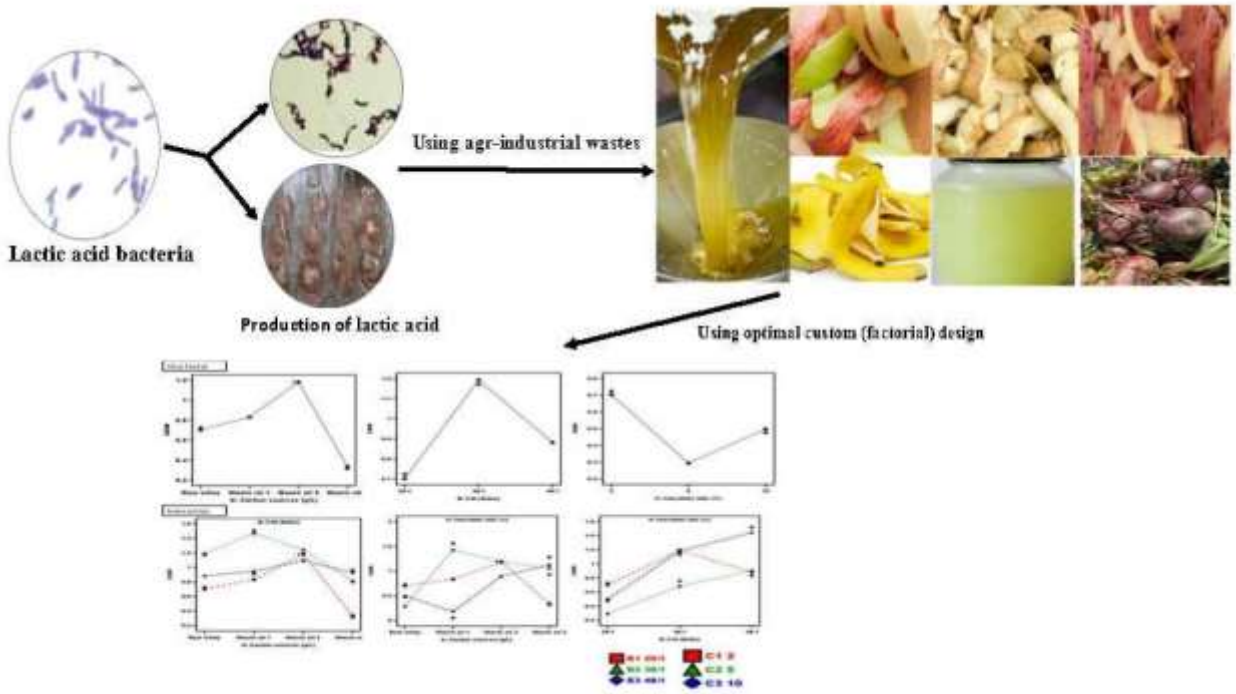
2 Department of Poultry Production, Faculty of Agriculture, Ain Shams University, Hadayek Shubra, Cairo 11241, Egypt

3Department of Food Science, Faculty of Agriculture, Ain Shams University, Hadayek Shubra, Cairo 11241, Egypt

[4]Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Hadayek Shubra, Cairo 11241, Egypt

Corresponding author e-mail: khadija_aboutaleb@agr.asu.edu.eg (Kh.A. Abou-Taleb) and maha_fawzy@agr.asu.edu.eg (M.F. Lotfy)

GRAPHICAL ABSTRACT



Abstract

Bioplastics made from renewable resources, such as contemporary biomass, have been developed more quickly as a result of global pollution from plastics made from petroleum, but it is still unclear how these materials will affect ecosystems. A common biopolymer that will make up 33% of the bioplastics produced in 2021 is polylactic acid (PLA). Forty-two bacterial isolates (soil, food wastes, and water wastes) were obtained, and 8 lactic acid bacteria were used for lactic acid (LA) production. These bacteria were classified into three categories, namely high, moderate and low LA producing bacteria which gave LA concentrations ranged from >1.0 g/L (3 bacteria), 0.5 to 1.0 g/L (21 bacteria) and 0.1 to 0.5 g/L (26 bacteria), respectively. The Sudan black staining method revealed that 16 bacteria out of 50 were capable of storing PLA granules, with three lactic acid bacteria referred to as *Lactococcus thermophilus*, *Lactobacillus rhamenose*, and *Lactobacillus retrieria* being the most efficient. The sodium dodecyl sulphate procedure was used to extract PLA from the selected LAB, and the results revealed that *L. rhamnose* was the most effective strain for producing both lactic acid and PLA. The most important fermentation parameters for lactic acid and polylactate polymer production were evaluated using an optimal custom (factorial) design. The interaction between three factors of selected potato oil waste, C/N ratio (48/1), and inoculum size (10%) resulted in an increase in lactic acid and polylactate polymer productivity by *L. rhamenose* L6, which reached 0.85 g/L, 0.96 g/L, and cell dry weight 2.33g/L, respectively.

Keywords: Bioplastic; accumulating bacteria; optimal custom(factorial) design; Lactic acid bacteria; polylactic acid; biodegradable and circular economy

1. Introduction

The increasing use of plastic-based materials has increased the demand for plastic globally, placing extra load on the system for disposing of waste **[1].** There is a great deal of concern in reducing the use of plastics made from petroleum, which pollutes the environment worldwide **[2].** Every year, more than eight million tonnes of plastic waste leak into the oceans, but this problem can be solved by creatively redesigning packaging materials **[3].** Traditional plastics like polyethylene and

polypropylene are affordable, lightweight, and versatile, but they also have a high resistance to corrosion, water, and bacterial decomposition, which makes them last for many years after being discarded. Due to its petrochemical origin, it turns into waste that is challenging to eliminate and, as a result, a significant environmental issue **[4].** In the 1940s, durable plastics were first produced **[5].** According to European Bioplastics **[6],** a significant amount of plastic is produced every year, with an estimated 370 million tonnes in 2021. This is because plastics have become an essential part of human life. The main causes of plastic accumulation in the environment are the non-biodegradable nature of the majority of petroleum-based plastics and a low recycling rate; as an example, only 9% of the total amount of plastic produced up until 2015 was recycled **[7].** Because bioplastics are being used, the remnants of packaging that wind up in drains or oceans will naturally degrade upon contact with water and environmental factors, destroying and emitting organic molecules that have no negative effects on the environment **[8].** The International Standards Organisation (ISO) defines bioplastics as polymeric structures made from carbon-rich wastes like used cooking oil, food scraps, and raw vegetables and fruits **[9].** A type of lactic acid derivative known as polylactic acid (PLA) is created using renewable resources like wheat, straw, corn, and sorghum, all of which are fully biodegradable **[10,11].** Microbes can break it down into water and carbon dioxide, making it environmentally friendly. One of the most widely used bioplastics today is PLA, but the process for this material to degrade is very specific and needs to take place in the right facilities; as a result, if it ends up in a landfill, it will stay there for a very long time, just like a regular plastic **[12].** A bio-based polymer called PLA can biodegrade under certain conditions, which will be discussed below, and does not pollute the environment when it does so **[13].** High elasticity modulus and stiffness, thermoplastic behaviour, biocompatibility, and effective moulding and shaping capabilities are all characteristics of PLA. Because of these characteristics, PLA has been successfully applied in agricultural applications, drug delivery systems, packaging materials, and surgical implant materials **[10,11].** The process of making PLA begins with the creation of LA and ends with its polymerization, with the formation of lactides acting as an intermediary step. The almost promising class of renewable

resource-based polymers are those based on LA (PLA) **[14].** The basic steps in the synthesis of PLAs are as follows: (i) microbial fermentation of LA, (ii) purification of LA and preparation of its cyclic dimer (lactide), and (iii) polycondensation of LA or ring-opening polymerization (ROP) of lactides **[15].** The effectiveness of PLA fermentation is largely dependent on the diversity of microbial species that produce it, including bacteria like Alcaligenes, Bacillus, Pseudomonas, Micrococcus, Rhodococcus, and lactic acid bacteria. These organisms also include fungi, yeast, cyanobacteria, and algae, as well as a number of other species **[16].** In the process of creating biodegradable plastics, PLAs are chosen as alternatives .

The purpose of this research was to find potential PLA-producing bacteria and assess PLA production using food waste and agricultural residues as carbon sources.

2. Materials and methods

2.1. Sample collection and isolation of lactic acid-producing bacteria

In order to isolate bacteria, various samples (soil, wastewater, and waste food) were obtained from various sources in Cairo, Egypt. The Microbiology Research Centre (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt, provided the four lactic acid bacterial strains *Lactobacillus plantarum* ATCC 14917, *Lactobacillus casei* DSM 20011, *Lactobacillus acidophilus* ATCC 20552, *Lactococcus thermophilus* DSM 20259. From the Food Technology Research Institute, Agriculture Research Centre in Giza, Egypt, four isolates of the lactic acid bacteria *Lactobacillus rhamenose* (L6), *Lactobacillus retrieria* (L7), *Lactobacillus bulgaricus* (L8), and *Lactobacillus plantarum* 2 (L9) were obtained. On de Man, Rogosa, and Sharpe (MRS) agar, the lactic acid bacteria were kept alive and used as a preservative **[17].**

One gram (soil and food waste samples) or one millilitre (wastewater samples) was added to 9 millilitres of sterilized distilled water. At 30°C, the samples were shaken for 30 min on a rotary shaker (150 rpm). Then, serial decimal dilutions were made, followed by streaking on nutrient agar plates. The plates were incubated for 24 h at 35°C under aerobic conditions. Streaking and purification were

repeated four times to ensure the purity of the isolates through morphological examination of the colonies and microscopic examination of the cells. All cultures of pure isolated bacteria were stored at -80 °C in 40% glycerol solution **[18].**

2.2. Screening for lactic acid production by bacterial strains and isolates

The Sudan Black B staining technique was used to screen bacterial isolates and strains for the presence of PLA granules. Two percentage of glucose was added to the nutrient agar medium. The plate was divided into equal sections, with bacterial isolates and strains distributed in each. The plates were incubated at 30 degrees Celsius for 24 h. Sudan Black B stain was made by combining 0.02 g powdered stain with 100 mL of 70% ethanol. Sudan Black B dye was spread over the plates after incubation and left undisturbed for 30 minutes. To remove the excess stain, the plates were washed with 96 percent ethanol. Colonies that were unable to integrate Sudan Black B appeared white, whereas PLA producers appeared bluish-black **[19].**

2.3. Lactic acid accumulation, pH, and cell dry weigh

To determine total titratable acidity (TA), 10 mL of weighed sample was placed in a conical flask, and 3 drops of phenolphthalein indicator were added and titrated with 0.1 mL of NaOH until a pink colour appeared. The TA value was recorded and expressed as a percentage of lactic acid using the following equation from **[20].**

$$\text{Lactic acid (mg/L)} = \frac{(0.1\ \text{M NaOH} \times \text{vol. of NaOH (in litter)} \times 90.08*) \times 1000}{(\text{vol. of the sampel})}$$

* 90.08 g/mol is the lactate molecular weight

A calibrated digital pH metre (HANNA Model HI -9321) was used to determine the pH value. After incubation, the bacterial culture was centrifuged for 15 min at 6,000 rpm. The pellet was dried at 55 °C. An empty pertiplate was weighed, then the plate containing dry cell weight (DCW) was weighed as a g/L **[21].**

2.4. Production and extraction of PLA

The announcing isolate's pure culture was inoculated in sterile nutrient broth media. After 24 hours at 30 °C, 1% (v/v) of the culture was transferred aseptically into a 250 mL conical flask containing 50 mL of mineral salts medium used for polylactic acid production medium **[19]** and it containing (in g/L): 20 glucose, $(NH_4)_2SO_4$, 1.0; KH_2PO_4,1.5; Na_2HPO_4. $12H_2O$. 9.0; $MgSO_4.7H_2O$, 0.2; pH 6.8 and 1 ml of trace elements solution ($FeSO_4.7H_2O$, 10; $ZnSO_4.7H_2O$, 2.25; $CuSO_4.5H_2O$, 1.0; $MnSO_4.4H_2O$, 0.5; $CaCl_2.2H_2O$, 2.0; $Na_2B_4O_7.10H_2O$, 0.23; $(NH4)_6\,Mo7O_{24}$, 0.1 and 35 % HCl 10 ml). It was then incubated for 72 h at 30 °C and 150 rpm. For 15 minutes, the culture broth was centrifuged at 5000 rpm. The pellet was dried after the supernatant was discarded. Recovering polylactic acid with sodium dodecyl sulphate (SDS): After being harvested, the cells were exposed to 10% SDS for 20 minutes at 100 °C. The pellets were centrifuged, cleaned, dried, and then extracted with chloroform at 60 °C for one hour. According to Bhuwal et al**. [22]**, the non-PLA cell matter was eliminated by filtration, and the dissolved PLA was separated from chloroform by evaporation. It was then twice washed with methanol, filtered out, and dried at 60-70 °C.

2.5. Effect of agro-industrial wastes on polylactic acid production

In batch culture, growth, and polylactic acid production were studied on different wastes as a sole carbon source and it was found their contained total carbon being i.e. treated whey (4.00%), potato peel (0.78%), sweet potato peel (4.18%), beet peel (17.00%), apple peel (10.40%), guava peel (8.92%), banana peel (15.00%), raw whey (4.30 %), and waste frying oils (fish, eggplant, and potato, chicken with 50.00%) were tested, have been previously determined by Abou-Taleb et al. **[23]**. To the initial carbon and nitrogen percentages in the basal medium, various carbon sources were added in an equal amount. Utilising the most effective carbon source found through earlier optimization experiments, various C/N ratios were tested.

2.6. Evaluation of the most significant fermentation parameters using optimal custom (factorial) design for lactic acid and poly-lactic acid production

Screening of the most significant physical factors affecting on biomass, lactic acid, and poly-lactate polymer production from *Lactobacillus rhamenose* L6 was investigated by optimal custom design using factorial of Design-Expert statistical software (Version 12, Stat-Ease, Inc., Minneapolis, MN, USA), as recommended by **Pourmortazavi *et al.* and Taran *et al.* [24, 25]**.

A total of 3 (n) independent variables, including carbon sources, C/N ratio, and inoculum size, were investigated, as shown in **Table (1).** The levels of independent variables used was 4 levels of carbon sources (raw whey, fish fried oil waste 1, eggplant fried oil waste 2, and potato fried oil waste 3), 3 levels of C/N ratio (29/1, 38/1 and 48/1), and 3 levels of inoculum size (2, 5 and 10 %)**.** In **Table (1),** a set of 29 experiments (runs) as a batch culture was conducted. The observed average was used as a response to the design after all trials were duplicated. Each row represented a trial run, and each column was an independent variable. ANOVA was used by Fisher's test to assess the effect of independent variables on the response and the significance of each variable influencing biomass production was determined by Student's t test with 95% confidence levels. The model involves *F*-value, *P*-value, main effect, standard division, mean, coefficient of determination (R^2), and % contribution. Factorial experimental design is based on the first-order model, which was determined by the following formula:

$$Y=B0+\Sigma Bixi \qquad \text{Equation (1).}$$

Where Y is the response (Cell dry weight), B0 is the model intercept and Bi was variables estimates. Effect of each variable was determined by the following equation:

$$E(Xi) = 2\,(+\Sigma M_i - \Sigma M_{i-}) / N \qquad \text{Equation (2).}$$

Where E (Xi) is the tested variable effect and Mi+ and Mi− represent biomass production from rials where variables (Xi) measured were present at high and low concentrations, respectively and N is the number of trials in **Equation (3).**

The standard error (SE) of the concentration effect was the square root of the variance of an

Table (1): D-optimal factorial design for screening significant independent variables for cell dry weight, lactic acid, and poly-lactate polymer synthesis from *Lactobacillus rhamenose L6*.

Runs No.		Carbon sources	C/N Ratio	Inoculum size (%)	Response		
					Lactic acid conc. (g/L)	CDW (g/L)	Poly-lactate polymer conc. (g/L)
Levels	1	Raw whey	29/1	2			
	2	Fish oil waste	38/1	5			
	3	Eggplant oil waste	48/1	10			
	4	Potato oil waste					
1		Raw whey	38/1	10	0.52	1.13	0.47
2		Potato oil waste	29/1	5	0.53	0.93	0.79
3		Fish oil waste	38/1	10	0.32	1.11	0.71
4		Raw whey	48/1	5	0.59	0.83	0.80
5		Eggplant oil waste	38/1	5	0.77	1.19	0.53
6		Fish oil waste	29/1	5	0.68	1.56	0.59
7		Eggplant oil waste	48/1	2	1.17	1.10	0.38
8		Potato oil waste	38/1	5	0.45	1.45	0.84
9		Eggplant oil waste	48/1	5	0.59	1.48	0.59
10		Eggplant oil waste	29/1	5	0.68	1.19	0.49
11		Potato oil waste	48/1	5	0.63	2.20	0.84
12		Fish oil waste	48/1	10	0.36	1.10	0.86
13		Fish oil waste	48/1	2	0.54	0.91	0.34
14		Raw whey	38/1	2	0.63	1.17	0.32
15		Potato oil waste	48/1	2	0.50	0.96	0.63
16		Fish oil waste	29/1	10	0.31	0.05	0.21
17		Potato oil waste	29/1	2	0.63	0.32	0.35
18		Raw whey	48/1	10	0.61	1.52	0.68
19		Potato oil waste	29/1	10	1.31	1.28	0.57
20		Potato oil waste	48/1	10	0.85	2.33	0.96
21		Eggplant oil waste	38/1	10	0.41	1.10	0.73
22		Raw whey	29/1	10	0.59	0.48	0.33
23		Raw whey	38/1	5	0.60	0.76	0.52
24		Potato oil waste	38/1	2	0.81	0.80	0.58
25		Eggplant oil waste	48/1	10	0.45	1.62	0.85
26		Fish oil waste	38/1	2	0.51	1.51	0.59
27		Fish oil waste	38/1	5	0.63	1.83	0.67
28		Eggplant oil waste	29/1	2	0.54	1.18	0.47
29		Raw whey	29/1	2	0.54	0.73	0.31

No., Number; C/N ratio, Carbon/Nitrogen; CDW, Cell dry weight; and Conc., concentration.

effect, and the significance level (*p*-value) of each concentration effect was determined using Student's t-test t (Xi) in **Equation (3).**

$$t(Xi) = E(Xi) / SE \qquad \textbf{Equation (3).}$$

Where E (Xi) is the variable Xi effect.

2.7. Statistical analysis

Duncan's Multiple Range Test was used to analyse data using the IBM® SPSS® Statistics programme version 19 at the 5% level **[26]**.

3. Results and discussion

3.1. Collection and isolation of lactic acid-producing bacteria

The bacteria were isolated by serially diluting the samples to 10^{-6} and cultured on a nutrient agar medium. As butyrate is a lipid molecule, logically, three mucoid colonies, one from three sources (soil, food, and water samples), were more focused during selection from many obtained colonies. Forty-two bacterial isolates were obtained, 21 (50%) from soil samples referred to as S1-S21, 13 (31%) from food wastes samples referred to as F1-F13, and 8 (19%) from water wastes samples referred to as W1-W13 **(Figure. 1)**. Out of these isolates, 58% of isolates were characterized to be long bacilli rods species, 23% were cocci species, 19% were short bacilli species.

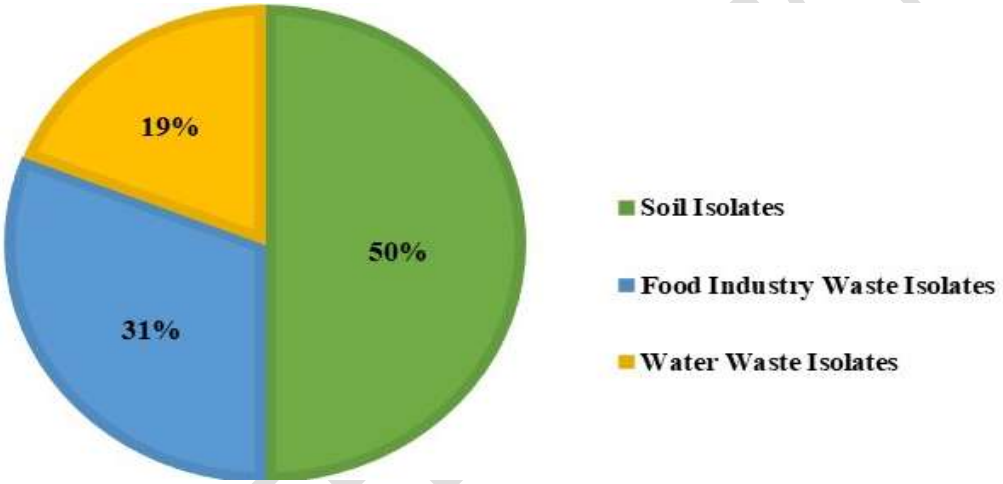


Figure.1 The numbers and percentage distribution of total isolates were isolated from different sources

In addition, eight lactic acid bacteria (LAB) strains and isolates as producers for lactic acid were used in this investigation. The tested LAB were stained with Gram staining and examined by light microscope (at 1000 x) including strains of *L. plantarum* ATCC 14917, *L. casei* DSM 20011, *L. acidophilus* ATCC 20552, *L. thermophilus* DSM 20259 and isolates of *L. rhamenose* L6, *L. retrieria* L7 and *L. bulgaricus* L8 and *L. plantarum* L9 were bacilli shaped, pairs or in short chains, Gram-positive, non-spore-forming bacteria, while *L. thermophilus* DSM 20259 strain was cocci shape in short chains, Gram-positive, a non-spore-forming bacterium **(Figure.2)**.

These results corresponding to **[27]** reported that *Bacillus* species were able to produce pure lactic acid reach to > 90%.

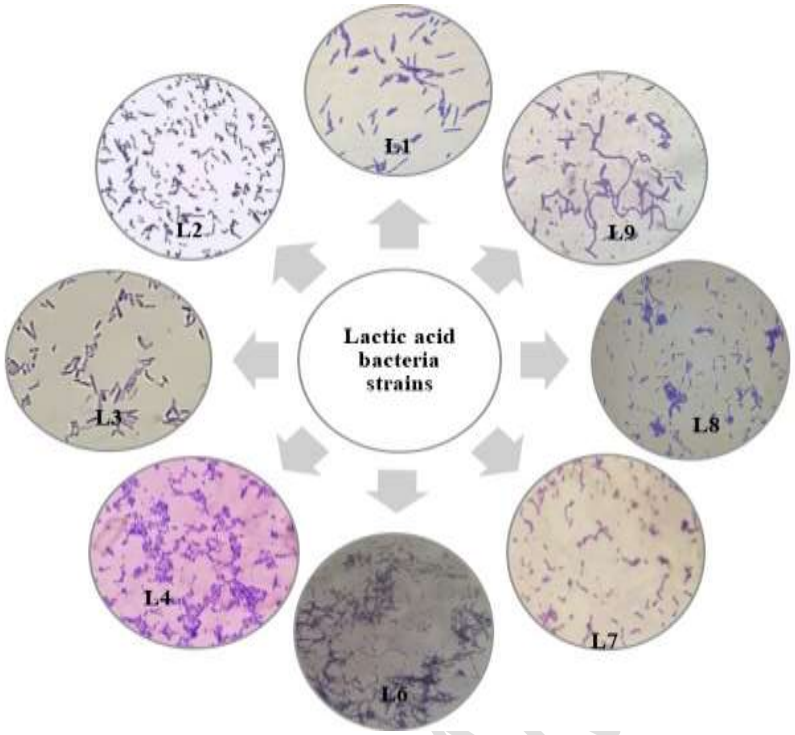


Figure. 2 Morphological properties of the tested lactic acid bacterial strains and isolates under light microscope (at 1000 x)

*L1, *Lactobacillus plantarum* ATCC 14917; *L2, *Lactobacillus casei* DSM 20011; *L3, *Lactobacillus acidophilus* ATCC 20552; *L4, *Lactococcus thermophilus* DSM 20259, *L6, *Lactobacillus rhamenose;* *L7, *Lactobacillus retrieria;* *L8, *Lactobacillus bulgaricus;* *L9, *Lactobacillus plantarum*.

3.2. Screening of lactic acid-producing bacteria

A number of high, moderate, and low lactic acid-producing bacterial strains and isolates according to lactic acid concentration (g/L) is shown in **Table.2**. A total of 3 lactic acid bacterial strains were identified as high lactic acid-producing bacteria lactic acid, so the concentration of lactic acid was >1.0. The numbers of moderate isolates for lactic acid production are 9,4, and 3 isolates, while the number of low isolates for lactic acid production was 12, 9, and 5 isolates are soil, food waste, and water waste isolates, respectively. Finally, the number of high, moderate, and low lactic acid-producing bacterial strains and isolates in all samples according to lactic acid concentration are 3, 21, and 26 isolates, respectively, have been represented in **Table.2**. The bacterial strains that produced the most lactic acid (LA) in the production medium were chosen for testing their potential for LA

production in various samples. Lactic acid bacteria (LAB) are Gram-positive bacteria that rely solely or primarily on carbohydrates for carbon **[28]**.

Table. 2 Number of high, moderate, and low lactic acid-producing bacterial strains and isolates according to lactic acid concentration

Sources of bacterial strains & Isolates	Lactic acid concentration (g/L)		
	High	**Moderate**	**Low**
LAB Strains and isolates	3	5	0
Soil isolates	0	9	12
Food waste isolates	0	4	9
Water waste isolates	0	3	5
Total strains and isolates	3	21	26

LAB, Lactic acid bacteria; *Low, acidity (as lactic acid) ranged from 0.1 to 0.5; **Moderate,** acidity (as lactic acid) ranged from 0.51 to 1.0; **High,** acidity (as lactic acid) is >1.0.

3.3. Polylactate detection

Fermentation or chemical synthesis are typically used to produce lactic acid. The best-studied polymer with at least one monomer synthesised by bacterial transformation is polylactic acid (PLA). While PLA typically has a molecular weight (MW) between 5 and 50 104 and a polydispersity between 1.8 and 2.6, obtaining a high MW for PLA was challenging **[29]**. Sudan Black is a lipophilic dye that binds strongly to lipids granules and others hence used in detecting and confirming the presence of PLA granules inside the organism. The granules are stained black, which can be seen under a microscope. Here, the most intensely stained colony. In this staining, the bacteria show positive Bluish Black and negative was red, which is displayed in **Figure. 3.**

Twenty of 50 bacterial isolates and strains showed positive for Sudan black staining and were able to produce PLA granules. Only 16 isolates and stains S1, S2, S3, S4, S5, S6, S7, S8, *Lactobacillus plantarum* ATCC 14917, *Lactobacillus casei* DSM 20011, *Lactobacillus acidophilus* ATCC 20552, *Lactococcus thermophilus* DSM 20259, *Lactococcus thermophilus* DSM 20259, *Lactobacillus rhamenose* L6, *Lactobacillus retrieria* L7 and *Lactobacillus bulgaricus* L8 and *Lactobacillus plantarum* L9 were selected for further study due to their high color intensity with

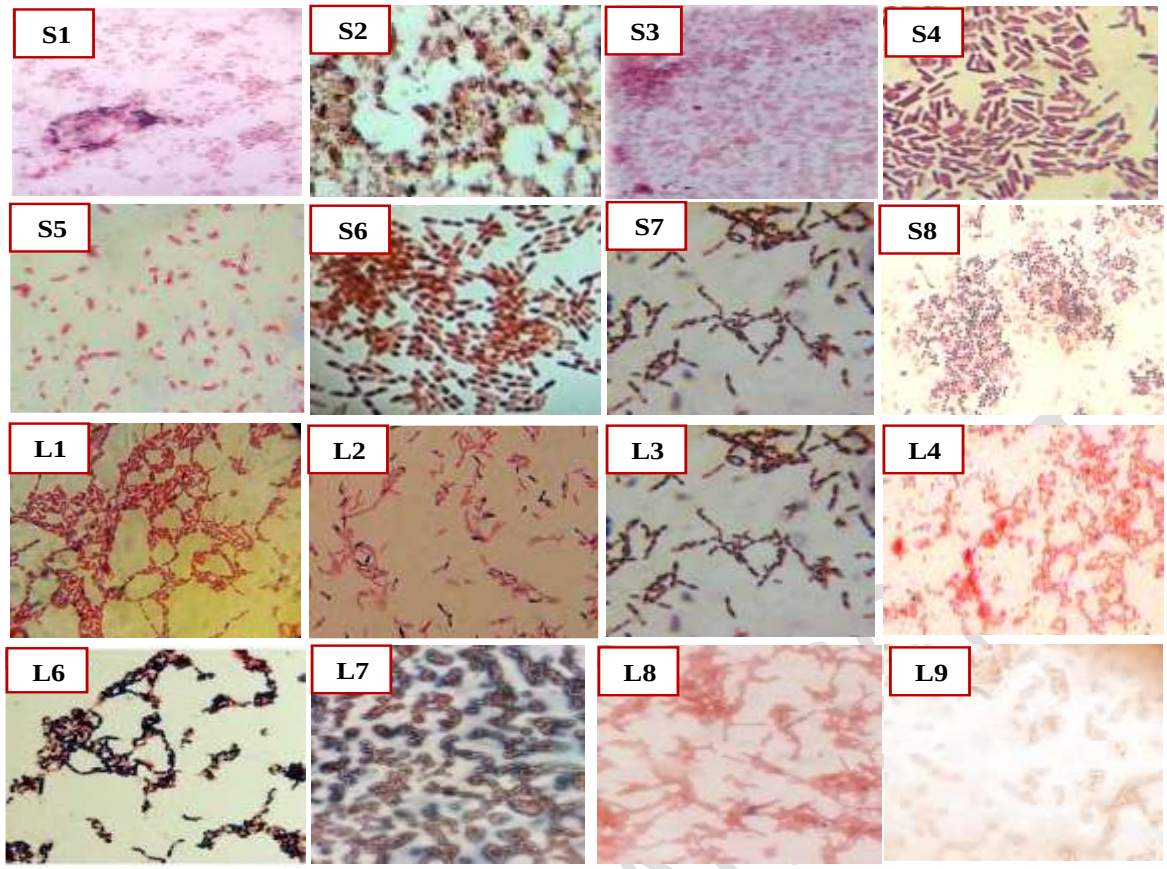


Figure.3 Poly-lactate storage cells of some bacterial isolates and strains stained with Sudan black staining under the light microscope (at 1000x)

*S1,2,3,4,5,6 were codes of some bacterial isolates. * L1-L9 were codes of Lactic acid bacterial isolates and strains (*L1, *Lactobacillus plantarum* ATCC 14917; *L2, *Lactobacillus casei* DSM 20011; *L3, *Lactobacillus acidophilus* ATCC 20552; *L4, *Lactococcus thermophilus* DSM 20259, *L6, *Lactobacillus rhamenose;* *L7, *Lactobacillus retrieria;* *L8, *Lactobacillus bulgaricus;* *L9, *Lactobacillus plantarum*). *The cells have poly-lactate that appeared Bluish Black. *The cells that don't have poly-lactate appeared red.

Sudan black. The accumulation of PLA by bacteria isolated from different soil samples and collection strains has been represented in **Figure.3**. PLAs like PHB are intracellular inclusions and can form approximately in all bacteria. The biosynthesized PLAs can reach up to 90% of the dry cell mass as a response to growth conditions deficiency [17]. Some microorganisms such as *Rolstonia*, *Alcaligenes*, *Micrococcus*, *Bacillus*, and *Pesudomonas* species *in* their wild types of forms can produce PLAs and PHAs between 50% and 80% of the dry cell mass **[30].**

Also, lactic acid concentrations were determined by titratable acidity. In general, lactic acid production and bacterial cell mass were increased over time for the eight tested strains during fermentation in the production medium. Among the eight lactic acid strains, the highest lactic acid

production and bacterial cell mass were recorded after 72 hrs. incubation of fermentation observed in **Figure**.**4**.

A total of three isolates and strain showing the highest growth rates and production of acidity were selected for microbioreactor fermentation. The results obtained that the lactic acid concentrations were 1.72, 2.61, and 2.15 g/L, While the pH values were 5.22, 3.83, and 4.83 for L7, L4, and L6 strains, respectively, under the same previous conditions of fermentation (**Figure.4**).

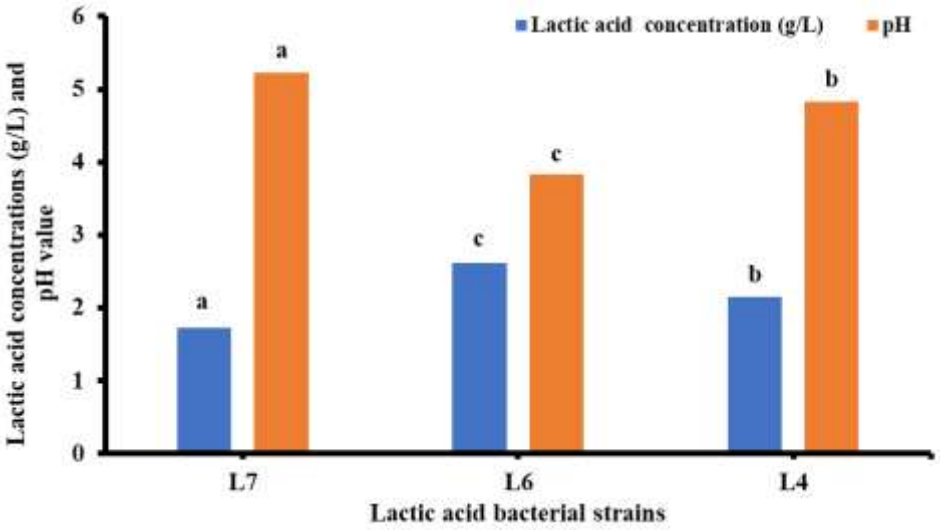


Figure. 4 The most efficient LAB were selected for lactic acid production

***(L7) =** *Lactobacillus retrieria,* ***(L6) =** *Lactobacillus rhamenose*, and ***(L4) =** *L Lactococcus thermophilus* DSM 20259 strain.•[a, b] Values with small letters in the same column having different superscripts are significant differences (at p≤ 0.05).

Lactic acid bacteria (LAB) are Gram-positive microorganisms, the main safe industrial-scale producers of lactic acid (LA). The glycolysis pathway produces LA under anaerobic conditions, and this compound can be produced from hexoses and pentoses LAB metabolism pathways. LA production yield and productivity depend on pH (3.5–9.6), temperature (5–45 °C), nutrients presence (such as amino acids, peptides, nucleotides, and vitamins), and the LAB strain producers used (so far have been used strains belonging to the genus *Leuconostoc, Lactococcus, Lactobacillus, Pediococcus, Enterococcus, Streptococcus, Vagococcus, Aerococcus, Carnobacterium, Tetragenococcus, Oenococcus* and *Weissella*) **[31**, **32]**. However, LAB species, including

L*actobacillus, Lactococcus, Leuconostoc, Streptococcus, and Pediococcus*, are also used as starter cultures in industrial food fermentations. Among LAB strains, *Lactobacillus* strains have great commercial importance due to high acid tolerance, high yield, and productivity, and can be engineered for the selective production of L/D-lactic acid **[33]**. So, LA is an organic molecule that is in growing demand worldwide due to its applications associated with the production of polylactic acid (PLA) **[33]**.

3.4. Agro-industrial materials

Agro-industrial and Food waste contain a high amount of carbohydrate, which causing it suitable as a substrate for lactic acid fermentation, numerous studies stated food waste was suitable for lactic acid production. The results showed that *Lactobacillus rhamenose* L6 had the highest lactic acid production (2.61 g/L). However, no significant differences were observed for the analyzed samples in the case of lactic acid production in the rest isolates, and strains were collected. The *Lactobacillus rhamenose* L6 was selected for fermentation using standard culture medium. This strain presented some of the highest lactic acid (LA) values during growth and had the capacity to ferment some sugars commonly present in agro-industrial residuals, such as treated whey, potato peel, sweet potato peel, beet dregs, apple dregs, guava dregs, banana dregs, raw whey, and oils wastes **(Figure.5)**.

LA production by *Lactobacillus rhamenose* L6 was 0.36, 0.615, 0.125, 0.125, 1.014, 0.215, 0.26, 0.62, 0.885, 0.945, 1.35 and 0.63 g/L with initial cell dry weight 0.0421, 0.065, 0.035, 0.059, 0.085, 0.034, 0.0817, 0.0889, 0.226, 0.183, 0.3114 and 0.082 g/L by using production medium contained treated whey, potato peel, sweet potato peel, beet dregs, apple dregs, banana dregs, guava dregs, raw whey, fish oil waste, Eggplant oil waste, potato oil waste, and oil waste 4, respectively **(Figure.5 a, b)**.

The highest lactic acid concentration was 1.35g/L with medium containing oil waste 3, and the lowest concentration was 0.125 g/L (sweet potato peel and beet dregs wastes) compared to the control treatments (glucose) that produced 2.49 g/L lactic acid **(Figure.5 a)**.

The fermentation experiments showed that higher production of lactic acid was obtained when the mineral salt medium contained raw whey, fish oil waste, Eggplant oil waste, and potato oil waste as sources of carbon when compared to the residual wastes used (treated whey, potato peel, sweet potato peel, beet dregs, apple dregs, guava dregs, and banana dregs).

Approximately 90% of the total lactic acid produced worldwide is made by bacterial fermentation, and the remaining portion is produced synthetically by the hydrolysis of lactonitrile **[34].** The fermentation processes to obtain lactic acid for sugars can be classified according to the type of bacteria used **[35]**. For cost-effectiveness in lactic acid production, the selected cheap raw materials should have the properties to produce high yield, high productivity, less by-product formation, and little or no contamination **[36].** The most common low-cost raw materials used in lactic acid production are agricultural wastes and food industry by-products **[37, 38]**.

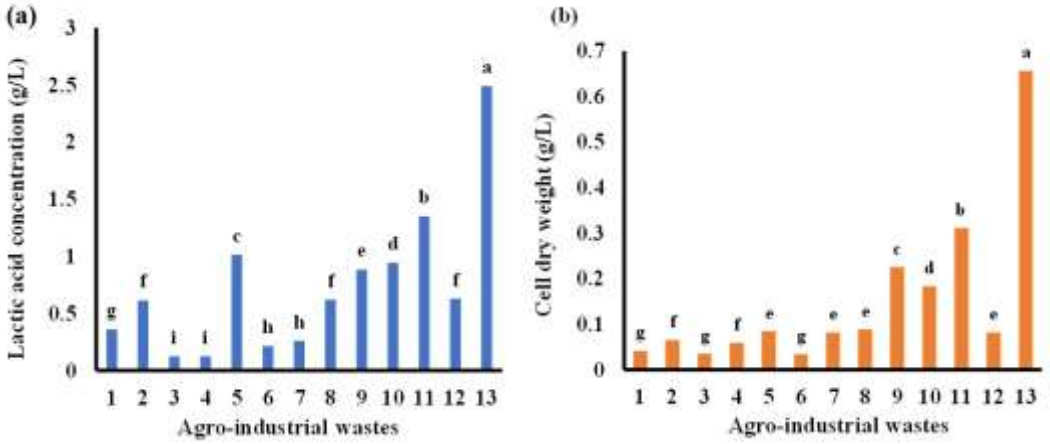


Figure.5 Cell dry weight and lactic acid production by *Lactobacillus rhamenose* L6 isolate using agro-industrial wastes under submerged fermentation

*1,Treated Whey; *2, Potato Peel; *3, Sweet Potato Peel; *4, Beet Dregs; *5, Apple Dregs; *6, Banana Dregs; *7, Guava Dregs; *8, Raw Whey; *9, Fish oil waste; *10, Eggplant oil waste; *11, Potato oil waste; *12, Oil Waste 4; and *13,Control (Glucose). • [a, b] Values with small letters in the same column having different superscripts are significant differences (at $p \leq 0.05$).

3.5. Evaluation of the most significant fermentation parameters using optimal custom(factorial) design for lactic acid and poly-lactic acid production

Evaluation of some environmental requirements of microorganisms is an important step for the development of biological processes. Improvement studies involving a one-factor approach at a

time are tedious, do not focus on studying the effect of interaction between factors, and may result in imprecision of results. Whereas statistical methodologies are generally preferred due to their advantages **[39].** Statistically designed experiments reduce error in determining the effect of parameters in an economical **[40]**.

Optimal custom (factorial) statistical design was performed to screen out the factors contributing to produce poly-Lactic acid by *Lactobacillus rhamenose* L6 from agro-industrial using submerged culture technique. Three variables carbon sources, C/N ratio, and inoculum size were tested for enhancing the *Lactobacillus rhamenose* L6 poly-lactic production (**Table.1).**

Data in **Table.1** showed a wide variation in poly-lactate polymer of *Lactobacillus rhamenose* L6, ranging from 0.21 to 0.96 g/L. The maximal poly-lactate polymer (0.96 g/L) was achieved during run number 20 with high levels of cell dry weight (2.33 g/L) and lactic acid concentration was (0.85 g/L). Whereas the lowest *Lactobacillus rhamenose* L6 lactic acid, poly-lactate polymer, and cell dry weight (0.31, 0.21, and 0.05 g/L, respectively) was recorded in run number 16.

Analysis of variance (ANOVA) through Fisher test was used to evaluate the effect of independent variables on the response and the significant results were identified by a p-value < 0.05. The model F-value of 4.91, 8.21, and 4.55 implies that the model is significant for lactic acid, cell dry weight, and poly-lactate polymer production from *Lactobacillus rhamenose* L6 isolate, respectively have been tabulated in **Table.3.** The smaller p-value indicates the high significance of the corresponding coefficient **[41]**.

The analyzed results in **Table.3** suggested that out of 3 independent variables, all variables of carbon sources, C/N ratio, and inoculum size were significantly affected (p-value= 0.019 & 0.03, 0.047 & 0.015, and 0.028 & 0.010) of lactic acid and poly-lactate polymer production, respectively. While two variables (C/N ratio and inoculum size) were significantly affected (p-value= 0.002 & 0.020, respectively) for cell dry weight. The standard division and mean were 0.11, 0.19, and 0.10 and 0.61, 1.16, and 0.58 for lactic acid, cell dry weight, and poly-lactate polymer production.

Table (3): Statistical analysis of variance (ANOVA) design for improving cell dry weight, lactic acid and polymer synthesis from *Lactobacillus rhamenose* L6 utilizing a D-optimal factorial design.

Source	Lactic acid conc.					CDW					Poly-lactate polymer conc.				
	Sum of Squares	df	Mean Square	*F*-value	*p*-value	Sum of Squares	df	Mean Square	*F*-value	*p*-value	Sum of Squares	df	Mean Square	*F*-value	*p*-value
Model	**1.27**	**23**	**0.056**	**4.91**	**0.042***	**6.70**	**23**	**0.292**	**8.21**	**0.014***	**1.06**	**23**	**0.046**	**4.55**	**0.049***
A-Carbon sources	**0.30**	**3**	**0.100**	**8.98**	**0.019***	**0.51**	**3**	**0.170**	**4.78**	**0.062**	**0.22**	**3**	**0.072**	**7.15**	**0.030***
B-C/N	**0.14**	**2**	**0.068**	**5.99**	**0.047***	**2.05**	**2**	**1.030**	**28.91**	**0.002***	**0.22**	**2**	**0.109**	**10.78**	**0.015***
C-Inoculum size	**0.18**	**2**	**0.090**	**7.98**	**0.028***	**0.68**	**2**	**0.339**	**9.53**	**0.020***	**0.27**	**2**	**0.137**	**13.54**	**0.010***
AB	**0.32**	**6**	**0.053**	**4.66**	**0.056**	**0.44**	**6**	**0.074**	**2.08**	**0.219**	**0.07**	**6**	**0.012**	**1.19**	**0.434**
AC	**0.26**	**6**	**0.043**	**3.77**	**0.083**	**2.12**	**6**	**0.353**	**9.93**	**0.012***	**0.09**	**6**	**0.015**	**1.45**	**0.351**
BC	**0.18**	**4**	**0.046**	**4.03**	**0.079**	**0.38**	**4**	**0.094**	**2.65**	**0.157**	**0.11**	**4**	**0.027**	**2.68**	**0.154**
ABC	**0.39**	**12**	**0.032**	**0.53**	**0.779**	**2.19**	**12**	**0.183**	**0.66**	**0.764**	**0.27**	**12**	**0.023**	**5.19**	**0.101**
Residual	**0.06**	**5**	**0.011**			**0.18**	**5**	**0.036**			**0.05**	**5**	**0.010**		
Cor Total	**1.33**	**28**				**6.88**	**28**				**1.11**	**28**			

Fit Statistics

Response	Std. Dev.	Mean	C.V. %	R^2	Adjusted R^2	Predicted R^2	Adeq. Precision
Lactic acid conc.	0.11	0.61	17.43	0.96	0.76	0.6	10.79
CDW	0.19	1.16	16.19	0.97	0.86	0.92	13.40
Polymer conc.	0.10	0.58	17.17	0.95	0.74	0.63	8.19

Conc.: concentration, CDW: Cell dry weight.

df: Degree of freedom, *P*: corresponding significance level, *F*: corresponding significance level, Std. Dev.: Standard Deviation, C.V: The coefficient of variation, R^2: Determination coefficient, Adj.: Adjusted, Pred.: Predicted, Adeq.: Adequate. *Significant at 0.05 level.

Adequate precision measures the signal-to-noise ratio. The ratio was 10.79, 13.40, and 8.19 for lactic acid, cell dry weight, and poly-lactate polymer production, which was greater than 4, it was desirable and indicates an adequate signal. Data also indicated that the R^2 was high determination ranged from 0.95 to 0.97, which means that 95 to 97 % of the total variation was explained by the model and predicted R^2 of 0.60, 0.92, and 0.63 is in reasonable agreement with the adjusted R^2 of 0.76, 0.86, and 0.74 for lactic acid, cell dry weight, and poly-lactate polymer production, respectively. In addition, the coefficient of variation (C.V) of each lactic acid, cell dry weight, and poly-lactate polymer production was 17.43, 16.19, and 17.17%, So, it was

concluded that the actual values were compatible with the predicted values, suggesting that the data matched the model well (**Figure. 6).**

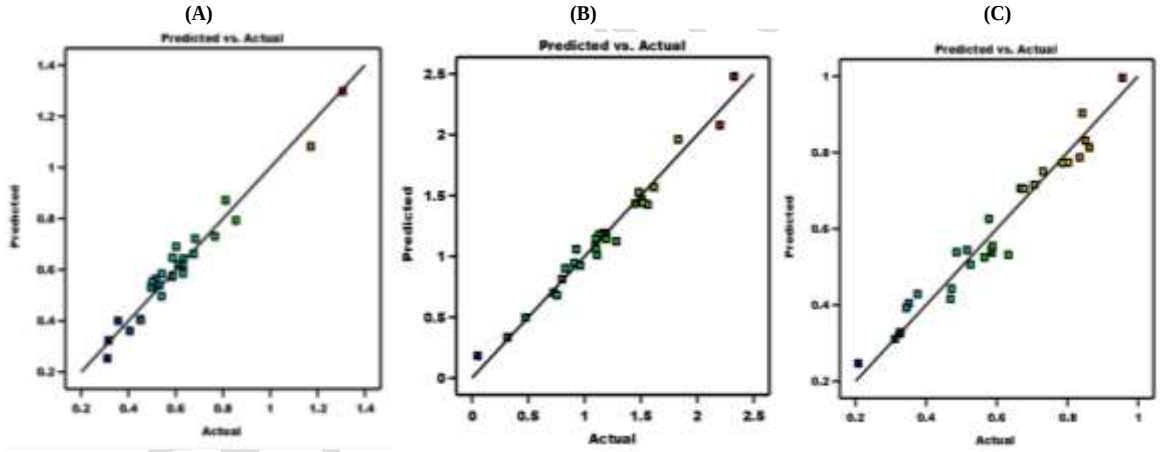


Fig. (6): The actual and predicted values of optimal custom (factorial) design for poly-lactate polymer production for *Lactobacillus rhamenose* L6.

Lactic acid concentration (A), cell dry weight (B), and polymer concentration (C).

In an optimal custom design, one factor and its interaction with another were also systematically estimated for the best biomass production shown in the models in (Figures. 7–

9). When one factor was influenced by another, there was an interaction between the factors as shown by two non-parallel lines. Factors were presented in parallel lines but did not interact.

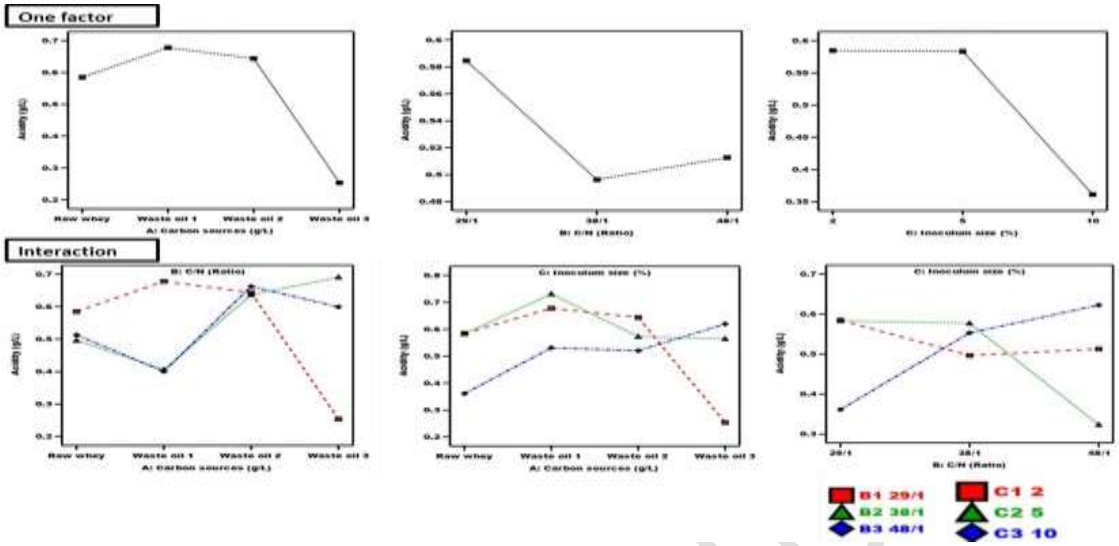


Figure. 7 The model graph at one-factor and interaction between two factors for lactic acid production from *Lactobacillus rhamenose* L6

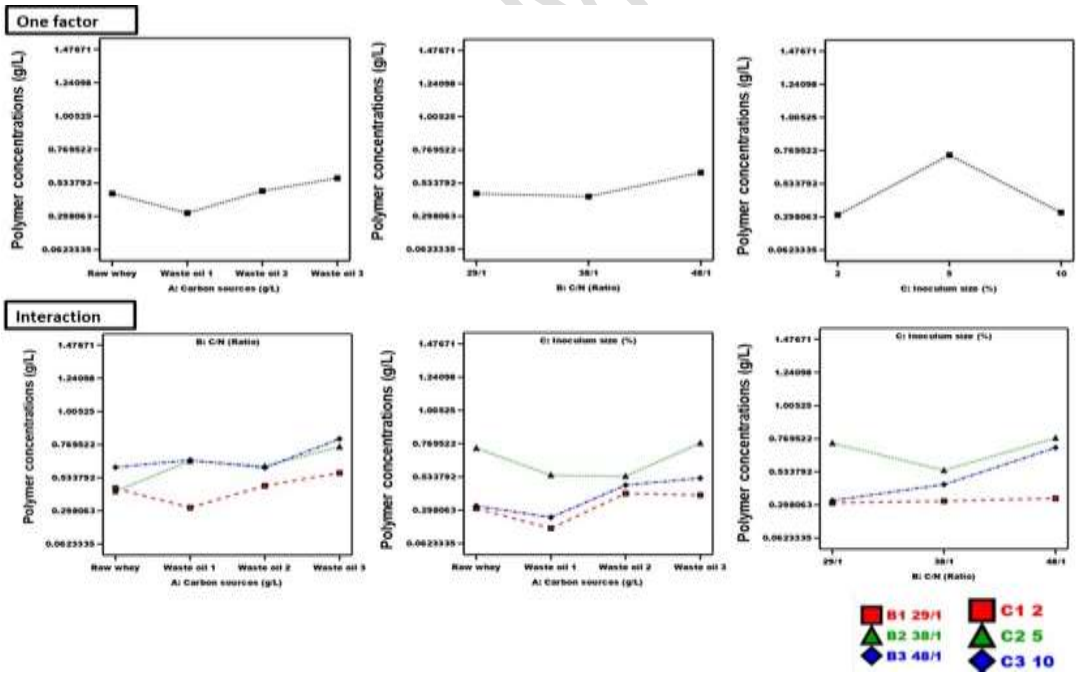


Figure. 8 The model graph at one-factor and interaction between two factors for cell dry weight of *Lactobacillus rhamenose* L6.

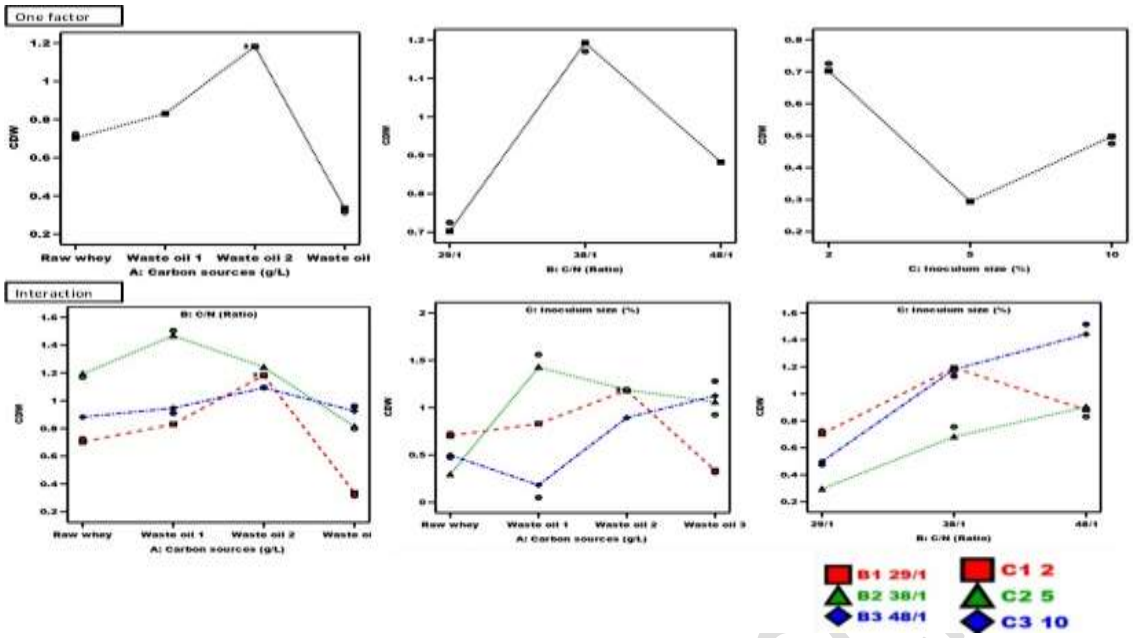


Figure.9 The model graph at one-factor and interaction between two factors for polymer production from *Lactobacillus rhamenose* L6

The equation for the ideal custom design (first order model) for *L. rhamenose* L6 poly-lactate grown in an industrial setting was discovered using Design-Expert and is as follows:

Y *Lactobacillus rhamenose* L6 lactic acid= +0.617 - 0.104 (lactic acid concentration) - 0.063 (cell dry weight) - 0.070 (polymer concentration) + 0.060 (lactic acid concentration * cell dry weight) + 0.045 (lactic acid concentration *polymer concentration) + 0.092 (cell dry weight *polymer concentration) **Eq. (4).**

Y *Lactobacillus rhamenose* L6 cell dry weight=+1.16 +0.050 (lactic acid concentration) - 0.348 (cell dry weight) + 0.149 (polymer concentration) -0.018 (lactic acid concentration * cell dry weight) + 0.142 (lactic acid concentration* polymer concentration) + 0.034 (cell dry weight * polymer concentration) **Eq. (5).**

Y poly-lactate polymer=+0.581-0.079 (Lactic acid concentration)-0.124 (cell dry weight)+0.098 (polymer concentration) +0.082 (lactic acid concentration* cell dry

weight) -0.017 (lactic acid concentration * polymer concentration) +0.027 (cell dry weight * polymer concentration) **Eq. (6).**

Finally, PLA has a wide range of uses. PLA is a crucial product for the circular economy and bio-based industries as well. Because of this, there has been a significant rise in demand for it on the global market recently, with a compound annual growth rate (CAGR) of 18.7% from 2019 to 2025. The United States, China, and Western Europe are the three biggest lactic acid markets in the world. The size of the global LA market was estimated at USD 2.7 billion in 2020, and it is anticipated to grow at a CAGR of 8.0% from 2021 to 2028 **[41]**.

5. Conclusion

An excellent substitute that can reduce the excessive use of non-biodegradable plastics made from petroleum are bioplastics made of polylactic acid. The capacity of Lactobacillus rhamenose L6 to store polylactic acid polymer inside its cells and produce lactic acid served as a key indicator for the amount of waste that can be recycled in the production of environmentally friendly bioplastic polymers.

Conflict of Interest

The authors declare no conflict of interest.

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References

[1] Rahman A. and Miller C.D. Microalgae as a Source of Bioplastics. (2017). In *Algal Green Chemistry: Recent Progress in Biotechnology*/*Edited by Rajesh Prasad Rastogi, Datta Madamwar, Ashok Pandey*; Rastogi R.P., Madamwar D. and Pandey A., Eds.; Elsevier: Amsterdam, The Netherlands, 121–138.

[2] Rastogi R.P., Madamwar D. and Pandey A. (2017). Algal Green Chemistry: Recent Progress in Biotechnology; Elsevier: Amsterdam, The Netherlands.

[3] MacArthur E. Beyond plastic waste. (2017). *Sci., 358*, 843. DOI: 10.1126/science.aao6749

[4] Valero-Valdivieso M.F., Ortegón Y. and Uscategui Y. (2015). Bioplastics: Advances and perspectives. Dyna 80: 171-180.

[5] Chia R.W., Lee J-Y., Kim H. and Jang J. (2021) Microplastic pollution in soil and groundwater: a review. *Environ. Chem. Lett.*, 19:4211–4224. DOI: 0.1007/s10311-021-01297-6.

[6] European Bioplastic. Market—European bioplastics. europeanbioplastics.org. (2022).

[7] Almeshal I., Tayeh B.A., Alyousef R., Alabduljabbar H., Mustafa M.A. and Alaskar A. (2020). Use of recycled plastic as fine aggregate in cementitious composites: a review. *Constr. Build Mater.* 253:119146. DOI: 10.1016/j.conbuildmat.2020.119146.

[8] Calabrò P.S and Grosso M. (2018). Bioplastics and waste management. *Waste Manage*. 78: 800-801. DOI: 10.1016/j.wasman.2018.06.054

[9] Endres H.J. (2019). Bioplastics. Adv. *Biochem. Eng. Biotechnol.,* 166: 427-468. DOI: 10.1007/10_2016_75

[10] Adsul M.G., Varma A.J., and Gokhale D.V. (2007). Lactic acid production from waste sugarcane bagasse derived cellulose. *Green Chem.* 9, 58–62. DOI: 10.1039/B605839F

[11] Singhvi M., Joshi D., Adsul M., Varma A. and Gokhale D. (2010). d-(-)-Lactic acid production from cellobiose and cellulose by *Lactobacillus lactis* mutant RM2-24. *Green Chem.*, *12*, 1106–1109. DOI: 10.1039/B925975A

[12] Maitz M.F. (2015). Applications of synthetic polymers in clinical medicine. *Biosurf. Biotribol*., 1: 161-176. DOI: 10.1016/j.bsbt.2015.08.002

[13] Qi X., Ren Y. and Wang X. (2017). New advances in the biodegradation of poly(-lactic) acid. *Int. Biodeterior. Biodegrad*., 117: 215-223. DOI: 10.1016/j.ibiod.2017.01.010

[14] Auras, R., Lim, L.T., Selke, M. and Tsuji, H. (2010). Poly (LA): Synthesis, Structures, Properties, Processing, and Application. Hoboken N.J.: John Wiley & Sons Inc.

[15] Singhvi M. and Gokhale D. (2013). Biomass to biodegradable polymer (PLA). *RSC. Adv.*, 3, 13558–13568. DOI: 10.1039/C3RA41592A

[16] Mikkili I., Karlapudi A.P. and Venkateswarulu T.C. (2014). Isolation, screening and extraction of polyhydroxybutyrate (PHB) producing bacteria from sewage sample. *Int. J. Pharm. Tech. Res.*, 6(2): 850-857.

[17] Power D.A. and Johnson J.A. (2009). Difco™ & BBL™ Manual. 2nd ed. Becton, Dickinson and Company, Sparks, Maryland 21152.

[18] Kim B.S., Lee S.C., Lee S.Y., Chang H.N., Chang Y.K. and Woo S.I. (1994). Production of PHB acid by fed-batch culture of *Alcaligenes eutrophus* with glucose concentration control. *Biotechnol. Bioeng*. **43**, 892–898. DOI: 10.1002/bit.260430908

[19] Olorunnisomo O., Ososanya T. and Adedeji O. (2014). Homogenization of milk and its effect on sensory and physico-chemical properties of yoghurt. *Afr. J. Food Sci.* 8, 465–470.

[20] Du G., Chen J., Yu J. and Lun S. (2001). Continuous production of poly-3-hydroxybutyrate by *Ralstonia eutropha* in a two stage culture system. J. Biotech., 88, 59-65.

[21] Jiang Y., Song X., Gong L., Li P., Dai C. and Shao W. (2008). High poly (-hydroxybutyrate) production by *Pseudomonas fluorescens* A2a5 from inexpensive substrates. *Enzym. Microb. Technol.*, 42:167-172. DOI: 10.1016/j.enzmictec.2007.09.003

[22] Bhuwal A.K., Singh G., Aggarwal N.K., Goyal V. and Yadav A. (2013). Isolation and screening of polyhydroxyalkanoates Producing Bacteria from pulp, paper, and cardboard industry wastes. *Int. J. Biomater.* 2013, 752821. DOI: 10.1155/2013/752821

[23] Abou-Taleb K.A., Mashhoor W.A., Nasr S.A. and Sharaf M.S. (2012). Exploitation of some agricultural by-products and wastes for vitamin B_{12} production by bacteria. *J. Biol. Chem. Environ. Sci.* 7, 77–91.

[24] Pourmortazavi S.M., Hajimirsadeghi S.S. and Rahimi-Nasrabadi M. (2009). Statistical optimization of condition for synthesis lead sulfide nanoparticles, *Mater. Manuf. Process*, 24:5, 524-528.

[25] Taran M., Rad M. and Alavi, M. (2017). Antibacterial activity of copper oxide (cuo) nanoparticles biosynthesized by *Bacillus* sp. FU4: Optimization of experiment design, *Pharm. Sci.*,23(3), 198-206. DOI: 10.15171/PS.2017.30

[26] Bryman A. and Cramer D. (2011). Quantitative data analysis with IBM spss 17, 18 and 19. 1st Edn., Taylor and Francis Group, United Kingdom, ISBN-13: 97802031 80990, Pages: 408.

[27] Zhang C., Zhou C., Assavasirijinda N., Yu B., Wang L. and Ma, Y. (2017). Non-sterilized fermentation of high optically pure d-lactic acid by a genetically modified thermophilic *Bacillus coagulans* strain. *Microb. Cell Fact.* 16, 213. DOI: 10.1186/s12934-017-0827-1

[28] George F., Daniel C., Thomas M., Singer E., Guilbaud A., Tessier F. J., and Foligné B. (2018). Occurrence and dynamism of lactic acid bacteria in distinct ecological niches: A multifaceted functional health perspective. *Front. Microbiol.*, 9, 2899. DOI: 10.3389/fmicb.2018.02899

[29] Zenkiewicz M., Richert J., Rytlewski P., Moraczewski K., Stepczynska M. and Karasiewicza T. (2009). Characterisation of multi-extruded poly(lactic acid). *Polym. Test.* 28, 4, 412-418. DOI:10.1016/j. polymertesting.2009.01.012

[30] Pei L., Schmidt M. and Wei W. (2011). Conversion of biomass into bioplastics and their potential environmental impacts. In: Biotechnology of Biopolymers. Elnashar, M. (ed.), InTech, Coratia. Pp. 57-74.

[31] Kyl€a-Nikkil€a K., Hujanen M., Leisola M. and Palva A. (2000). Metabolic engineering of *Lactobacillus helveticus* CNRZ32 for production of purel-(þ)-lactic acid. *Appl. Environ. Microbiol*., 66, 3835–3841. DOI: DOI: 10.1128/AEM.66.9.3835-3841.2000

[32] Zheng J., Wittouck S., Salvetti E., Franz C.M.A.P., Harris H.M.B., Mattarelli P., O'Toole P.W., Pot B., Vandamme P., Walter J., Watanabe K., Wuyts S., Felis G.E., G€anzle M.G. and Lebeer S. (2020). A taxonomic note on the genus Lactobacillus: description of 23 novel genera, emended description of the genus *Lactobacillus Beijerinck* 1901, and union of Lactobacillaceae and Leuconostocaceae. *Int. J. Syst. Evol. Microbiol*. 70, 2782–2858. DOI: 10.1099/ijsem.0.004107

[33] Gupta B., Revagade N. and Hilborn J. (2007). Poly(lactic acid) fiber: an overview. *Prog. Polym. Sci.* 34:455–482. DOI: 10.1016/j.progpolymsci.2007.01.005

[34] John R.P., Nampoothiri K.M., and Pandey A. (2007). Fermentative production of lactic acid from biomass: An Overview on process developments and future perspectives. *Appl. Microbiol. Biotechnol*. 74, 524–534. DOI: 10.1007/s00253-006-0779-6

[35] Mladenovi´c D., Pejin J., Koci´c-Tanackov S., Radovanovi´c Ž., Djuki´c-Vukovi´c A., and Mojovi´c L. (2018). Lactic acid production on molasses enriched potato stillage by *Lactobacillus paracasei* immobilized onto agro-industrial waste supports. *Ind. Crops Prod.* 124, 142–148. DOI: 10.1016/j.indcrop.2018.07.081

[36] Dumbrepatil A., Adsul M., Chaudhari S., Khire J. and Gokhale, D. (2008). Utilization of

molasses sugar for lactic acid production by *Lactobacillus delbrueckii* subsp. *delbrueckii* mutant Uc-3 in batch fermentation. *Appl. Environ. Microbiol*. 74, 333–335. DOI: 10.1128/AEM.01595-07

[37] Vidra A., Tóth A.J. and Németh, Á. (2017). Lactic acid production from cane molasses. *Waste Treat. Recovery*, 2, 13–16. DOI: 10.1515/lwr-2017-0003

[38] Polylactic Acid Market Size Share and Trends. [(accessed on 17 April 2021)]; Available online: www.researchandmarkets.com

[39] Reedy J.P., Mitrou P.N., Krebs-Smith S.M., Wirfalt E., Flood A. and Kipnis V. (2008). Index-based dietary patterns and risk of colorectal cancer: the NIH-AARP diet and health study. *Am. J. Epidemiol.*, 168: 38–48. DOI: 10.1093/aje/kwn097

[40] Sharma D.C and Satyanarayana T.A. (2006). Amarked enhancement in the production of a highly alkaline and thermostable pectinase by *Bacillus pumilus* dcsr1 in submerged fermentation by using statistical methods. *Bioresour. Technol*., 97(5):727-33. DOI: 10.1016/j.biortech.2005.04.012

[41] Tanyildizi M.S., Ozer, D. and Elibol, M. (2005). Optimization of α-amylase production by *Bacillus* sp. using response surface methodology. *Process Biochem*. 40, 2291-2296. DOI: 10.1016/j.procbio.2004.06.018