ISOLATION OF PROMISING STRAINS OF *BACILLUS SUBTILIS* FOR THE PRODUCTION OF LOW MOLECULAR WEIGHT γ-PGA

Stephen D. Titus[1], Shuaibu M. Bala[2], Mohammed S. Aliyu[3] and Elewechi Onyike[2],
[1]Department of Biological Sciences, Taraba State University, Jalingo.
[2]Department of Biochemistry, Ahmadu Bello University, Zaria.
[3]Department of Microbiology, Ahmadu Bello University, Zaria.
Corresponding Author: titus.stephendio@gmail.com, titus.stephen@tsuniversity.edu.ng

Received: September 14, 2023 **Accepted:** November 28, 2023

Abstract: Poly-γ-glutamic (γ-PGA) acid is a multifarious biopolymer and one of the emerging biopolymers that is currently in the centre stage of active investigation. This is because of its unique non immunogenic, nontoxic, water solubility and retention, and availability of functional groups for attachments of other bioactive compounds. This research was aimed at isolating and characterizing strains of Bacillus subtills for the production of low molecular γ-PGA. The B. subtilis isolated from natural habitats (Soil samples were pooled from roots of Sorghum bicolor, water was obtain from Ahmadu Bello University Dan, and Daddawa, a local condiment was purchased from Samaru Market) in Zaria Kaduna State Nigeria. Three isolates of Bacillus subtilis were purified from the collected samples in which only the soil sample gave positive Biochemical inference for B. subtilis. The isolates were further characterized by 16S rRNA gene sequencing. The strains were then used for a preliminary production of poly-γ-glutamic acid in submerge broth. From the three purified isolates designated S1, S4 and S5. Highest yield of 251.67±30.00 mg/gds of γ-PGA by submerge fermentation was obtained from isolate S5. Which was used for solid state fermentation (reported elsewhere). The study shows that Isolate S5 is a suitable candidate showing potential for fermenting low molecular weight γ-PGA, holding promise for industrial applications.

Key words: *Bacillus subtilis*, isolate, Fermentation, Rice husk, Poly-γ-glutamic acid

Introduction

The search for better ways to improve the standard of living through development of new and safer technologies in the area of enzymes and industrial biopolymers in the production of useful medical and pharmaceuticals drivers such as hydrogels, drugs carriers, gene delivery vehicles and super absorbents is in the centre stage of modern-day research (Das *et al*., 2018; Ramazan, 2019). Poly γ-glutamic acid (γ-PGA) is one of such biopolymers (Zhang *et al.,* 2018). Poly γ-glutamic acid is a polyamide, anionic biopolymer composed mainly of D-and L-glutamic acid units connected via amide linkages between α-amino and γ-carboxylic acid groups of the monomers (Hsueh *et al*., 2017). It has adhesive, film forming, and moisture retention properties making it an interesting material for drug delivery/release, bio-adhesive, cosmetics, food, agriculture, and sewage treatment (Lin *et al*., 2016). Because of its wide applications, it has become one of the most interesting topics in biopolymer research (Ajayeoba *et al.,* 2019). Amongst other novel applications, it has the potential to be used for protein crystallization, as a soft tissue adhesive and a non-viral vector for safe gene delivery (Ogunleye *et al.,* 2015).

The aim of this study was to isolate and screen for a potential γ-PGA producing *B. subtilis* strains from environmental samples.

Materials and Methods

Chemicals and Equipment

All reagents and chemicals used in this research were of analytical and molecular grade purchased from Sigma Aldrich Missouri, USA; whereas primers and media are from Inqaba Biotec, West Africa and Titan Biotech Rajasthan, India respectively.

Some of the equipment used include; PCR thermocycler (Labnet MultiGene OptiMax), UV-VIS Spectrophotometer (722N, CGOLDENWALL), Microscope (LX300, Labomed, Inc.), Autoclave (YX-280, Healicon Medical Equipment), Centrifuge (Heraeus Labofuge 300, Fisher Scientific), pH meter (pH 009, Henan), Weighing balance (FA2004, China), Incubator (IH-150, Gallenkamp) and Water bath (SHA-C, China).

Samples and Sample Collection

Soil samples were collected from roots of *Sorghum bicolor* in a farm land behind Pensioners quarters, Hayin Dogo Samaru Zaria, Kaduna State, Nigeria. The water samples were obtained from the ABU dam at the University main campus, Samaru, Zaria, Kaduna State, Nigeria while *daddawa* was purchased from market women in Samara market Zaria, Kaduna State, Nigeria. The soil, water and *daddawa* samples were collected in clean and sterile sample containers which were properly labelled and conveyed to the laboratory for the isolation of *B subtilis*.

Isolation of microorganisms

Bacillus subtilis strains were isolated from the samples of water, soil as well as from the local soup condiment; *daddawa*. The isolations were carried out according to the method described by Kim *et al*. (2012). About10g of soil were taken aseptically and dissolved in 50 mL and 10 mL distilled water and thoroughly shaken to dissolve. The dissolved samples were incubated at 80°C for 10min. and allowed to cool. The samples were picked with a loop and streaked on separate plates. Colonies formed on the plates were picked and sub-cultured in nutrient agar plates until pure cultures were obtained (Kim *et al*., 2012). Colonies obtained after incubation were observed and the creamy, viscous rod-shaped bacterial colonies were selected and sub-cultured on nutrient agar and incubated for another 24 hours at 37^0C. Gram staining reactions and cell morphology from heat fixed smears was used to select Gram+ colonies based on the method of microbial identification described by Cowan and Steel (1966). Pure cultures of Gram+ were isolated and preserved on nutrient agar slants and stored at 4ºC.

Biochemical and microscopic characteristics of the isolates were carried out based on the method of microbial identification described by Cowan and Steel (1966); Olutiola *et al*. (2000); Fawole and Oso (2004); Cheesbrough (2006) thus;

Gram staining techniques:

A thin smear of each of the pure 24 hours old culture was prepared on clean grease-free slides, fixed by passing over gentle flame. Each heat-fixed smear was stained by addition of 2 drops of crystal violet solution for 60 seconds and rinsed with water. The smears were again flooded with Liquol iodine for 30 seconds and rinsed with water, decolourized with 70% alcohol for 15 secoonds and were rinsed with distilled water. They were then counter stained with 2 drops of Safranin for 60 seconds and finally rinsed with water, then allowed to air dry. The smears were mounted on a microscope and observed under oil immersion objective lens. Gram negative cells appeared pink or red while gram positive organisms appeared purple (Fawole and Oso, 2004).

Spore staining technique:

Heat-fixed smears of the organisms were prepared on separate slides and flooded with 5% Malachite green solution and steamed for 1 minute which was thereafter washed off with water and counter stained with 2 drops of Safranin solutions for 20 seconds. The slides were allowed to air dry and examined under oil immersion objective (100) lens revealing stained endospores (Cheesbrough, 2006).

Motility test:

Motility tests were carried out according to the method described by Olutiola *et al*., (2000). A sterile needle was used to pick a loop-full of 24 hours old cultures and stabbed onto nutrient agar in test tubes which were incubated at 37°C for 48 hours. Motility was confirmed by pulsating growth emerging from the stabbed margin (Olutiola *et al*., 2000).

Methyl red test:

Five millimetres of glucose phosphate broth (1 g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 mL distilled water) were dispensed in clean test tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 37°C for 48 hours. At the end of incubation, few drops of methyl red solution were added to each test and colour change was observed. A red colour indicates a positive reaction (Olutiola *et al*., 2000).

***Voges-proskaeur test*:**

Five millimetre of glucose phosphate broth (1 g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 mL distilled water) were dispensed in clean test tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 37°C for 48 h. After incubation, 6% α-naphhtol and 6% Sodium hydroxide were added to about 1 mL of the broth culture. A strong red colouration formed within 30 min indicates positive reaction (Olutiola *et al*., 2000).

Indole test:

Tryptone broth (5 mL) was placed into different test tubes after which a loopful of the isolates was inoculated into the test tubes, leaving one of the test tubes uninoculated to serve as control. The test tubes were then incubated at 37°C for 48 h. After incubation, 0.5 mL of Kovac's reagent was added and shaken gently; it was allowed to stand for 20 minutes to permit the reagent to rise. A red or red-violet coloured ring at the top surface of the tube indicates a positive result while yellow colouration indicates a negative result (Cheesbrough, 2006).

Citrate test:

This test detects the ability of an organism to use citrate as a sole source of carbon and energy. About 2.4 g of citrate agar was dissolve in 100 mL of distilled water. About ten millilitre (10 mL) of citrate medium was dispensed into each tube and covered, then sterilized and allowed to cool in a slanted position. The tubes were inoculated by streaking the organisms once across the surface. A change from green to blue indicates utilization of the citrate.

Oxidase test:

A piece of filter paper was soaked with few drops of oxidase reagent. Sterile inoculating loop was used to pick a colony of the test organism and smeared on the filter paper. If the organism is oxidase producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour (Cheesbrough, 2006).

The 16S rRNA gene sequencing was amplified using the following oligonucleotide primers; forward: (AGAGTTTGATCCTGGCTCAG) and reverse: (AAGGAGGTGATCCAGCCGCA) to amplify the full length of the 16S rRNA gene (Hengstmann, 1999).

The 16S rDNA PCR products were extracted from gel using gel extraction kit QIAquick Qiagen while Sanger DNA sequencing technique was used to sequence the amplicons extracted from the gel. Sequence analysis was performed with the sequences BLAST on the NCBI database (www.ncbi.nlm.nih.gov/blast) (Camacho *et al*., 2009).

Selection of Promising Isolate and Purification of Produce γ-PGA

The isolates were screened for their γ-PGA producing ability in submerged fermentation. A starter culture of each isolate was prepared by inoculating the isolates in Luria Broth (LB) and grown overnight (Cheesbrough, 2006). Poly-γ-glutamic acid was purified using ethanol precipitation method as modified by Bajestani *et al*. (2018). Poly-γ-glutamic acid was purified using ethanol precipitation method as modified by Bajestani *et al*., (2018). The solid-state fermentation (SSF) media was suspended in 10 ml distilled water and agitated using shaker for 40 minutes. The supernatant containing γ-PGA was removed by centrifuging at 1000 rpm for 15 minutes and decanted into fresh tubes. The supernatant was then centrifuged at 6000×*g* for 40min and the supernatant containing low molecular weight dissolved substance were discarded. To the pellets in the tubes, 1:4 volume of water to ethanol was then added. The resultant solution was allowed to stand for one day. Decanted suspension was then centrifuged again at 6,000×*g* for 40min and the resulting pellets were dried and suspended in 5 ml of distilled water. The suspension was further centrifuged at 6,000×*g* for 1 hour to obtain pallets of produced γ-PGA which was dried and stored at 4ºC for further analyses (Bajestani *et al*., 2018).

Data Analysis

All analyses were carried out in triplicates and expressed as mean± SD. One-way ANOVA ($p \leq 0.05$) were considered significant.

Results

Microscopic and Biochemical Characteristics of the isolates

Three isolates of *B. subtilis* were obtained from the samples collected for the study as identified by their colony morphology and biochemical characteristics of the microbial growth on streaked nutrient agar plated and were designated S1, S4, and S5 respectively as shown in Table 1.

Table 1: Biochemical and spore staining characteristics of the isolates

SN	Tests	S1	S4	S5
1	Motility	+	+	+
2	Oxidase	+	+	+
3	Methyl red	-	-	-
4	Voges-Proskauer	+	+	+
5	Indole	-	-	-
6	Citrates	+	+	+
7	Spore Staining	Endo	Endo	Endo

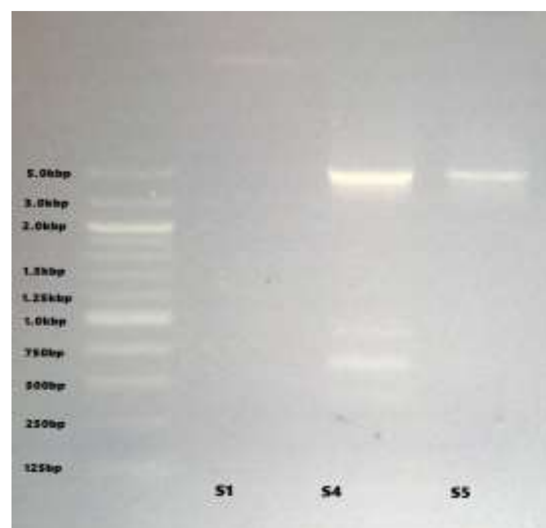

Figure 1: Electropherogram of isolates' 16S rRNA gene PCR products

Table 2: Sequence BLAST Analysis of Isolates' 16S rRNA Gene

S/N	Isolates	Matches	NCBI Accession	E value	Identity
1	S1	*Bacillus subtilis* 16S rRNA gene	MW 785886-785887	0	95.73%
2	S4	*Bacillus subtilis* 16S rRNA gene	MW805751	0	90.54%
3	S5	*Bacillus subtilis* 16S rRNA gene	MW 805756-805757	2.00E-174	91.04%

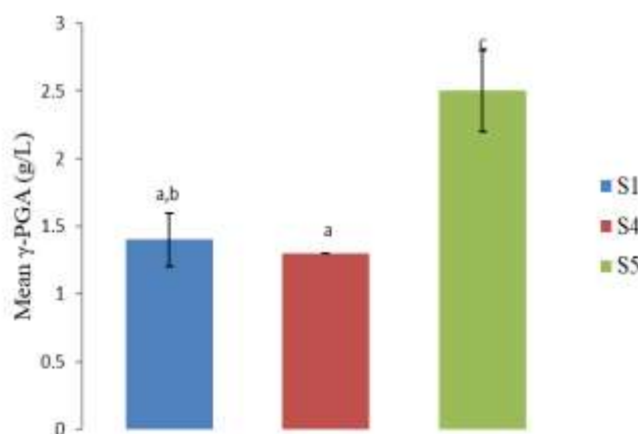

Figure 2: γ-PGA yields of Isolates in Submerged Broth

Discussions

The isolates' 16S rRNA PCR products gave band of about 3000bps (Figure 1). The sequence BLAST analysis on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed close similarity to *B. subtilis* with percentage identity scores of more than 90% (Table 2). The sequences are deposited on the NCBI gene bank database with the following accession numbers; S1 (MW 785886-785887), S4 (MW805751) and S5 (MW 805756-805757). Isolate S5 gave a significant higher yield of 2.50±0.30g/L of γ-PGA in broth media compared to isolate S1 and S4 with yield of 1.40±0.20 and 1.3±0.00g/L of γ-PGA (figure 2).

Conclusion

B. subtilis isolate S5 is a promising strain for the fermentation of low molecular weight γ-PGA. The yield of S5 was considered significantly higher and better at P≤0.05.

Conflict of Interest

The Authors declare no conflict of interest.

Acknowledgments

We want to acknowledge the Petroleum Technology Development Fund (PTDF) for their generous funding, the Staff of Departments of Biochemistry and Microbiology, Ahmadu Bello University, Zaria.

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