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DIVERSITY OF BACTERIA DURING FERMENTATION OF LIMABEAN INTO *DADDAWA*

Elizabeth Oluremi Farinde[*1], *Sumbo Henrietta Abiose*[2], *and Hezekiah Adekanmi. Adeniran*[2]

Address(es): Dr. Elizabeth Farinde,
[1]Product Development Programme, Institute of Agricultural Research and Training, PMB 5029, Ibadan, Nigeria. Phone: +2348075459882.
[2]Department of Food Science and Technology, Obafemi Awolowo university, Ile-Ife, Nigeria.

*Corresponding author: **osekinat@yahoo.co.uk**

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ABSTRACT

The diversity and succession of bacteria during the natural fermentation of lima bean (*Phaseolus lunatus*) to produce *daddawa* (a fermented condiment) was studied using molecular method (16SrRNA gene analysis) with a view to develop a framework for production of *daddawa* of consistence quality with starter culture of *Bacillus* species. Lima bean was fermented for 72 h, during which isolation of bacteria and extraction of DNA were carried out. The extracted DNA of the bacterial isolates was tested for quality using agarose gel electrophoresis. The results of the 16SrRNA gene analysis were matched with the existing similar sequences in data base. Twenty six (26) presumptive isolates of *Bacillus* obtained at 24 h interval during the natural fermentation process were identified. The result of the ratio of absorbances of the extracted DNA at 260 and 280 nm showed that 73% of the isolates had pure DNA while the result of the gel electrophoresis showed well defined bands of the amplicons for the isolates. The BLAST result identified the isolates as *Bacillus amyloliquefaciens, B. subtilis, B. pumilus, B. cereus* and *B. anthrasis* with *B. subtilis* been the most predominant. *Bacillus amyloliquefaciens, B. subtilis* and *B. pumilus* occurred through-out the fermentation process. The study established the identity of the important *Bacillus* species involved in fermentation of lima bean into *daddawa* using molecular technique. These major *Bacillus* species could further be tested and developed as potential starters for improved production of *daddawa* from lima bean.

Keywords: Limabean, *daddawa*, Fermentation, *Bacillus*, 16SrRNA gene analysis

INTRODUCTION

Daddawa also known as *iru*, among the Yorubas in South-west Nigeria, is a popular condiment used as taste and flavour enhancer in soup and dishes in Africa. *Daddawa* is traditionally produced from locust beans (*Parkia biglobossa*) seeds. The tree is a leguminous plant found in the Savannah region of Africa, South East Asia and South America **(Egwim *et al*., 2013)**. The tree is a perennial plant with pods ranging from pink, brown to dark brown, when matured. Nutritionally, African locust bean is an outstanding source of plant protein **(Elemo *et al*., 2011)**. However, locust bean trees are going into extinction from their natural habitat because they are not cultivated. Lima bean (*Phaseolus lunatus*) is an under-utilized legumes, whose seeds are good source of protein, dietary fibre, potassium, iron, copper, phosphorous, magnesium and thiamin (**WHFoods, 2010**). Previous study has shown that Lima bean could be used in production of *daddawa* (**Farinde *et al*., 2011**).

Biochemical method has been the conventional method for identification of isolates in fermentation process to produce *daddawa* **(Abiose *et al*., 1986; Barber *et al*., 1988; Achi, 1992; Barimalaa *et al*., 1994; Omafuvbe *et al*., 2000, 2002; Farinde *et al*., 2011**). This method may not account for minor microbial populations, stressed or injured cells that may be present in low number **(Fleet, 1999**). The biochemical method may not give a complete representation of food microbial community **(Kesmen *et al*., 2012**). In the last decades, interests in microbial ecology have increased due to advances in molecular biology such as advent of polymerase chain reaction and DNA sequencing (**Cocolin and Ercolini, 2009**). Succession of microorganisms involved in fermentation of African locust bean seeds using culture dependent techniques have been studied by various researchers (**Abiose *et al*.,1986; Achi, 1992; Omafuvbe *et al*., 2004; Adelekan and Nwadiuto,2012; Adewunmi *et al*., 2013**) and it has been shown that the major microorganisms involved in the fermentation process are the *Bacillus* species. Molecular genotyping techniques are considered to be effective and rapid tool for identification and characterization of *Bacillus* (**Morten *et al*., 2000; Miambi *et al*., 2003**).

These techniques include restriction fragment length polymorphism (RFLP) for the grouping and typing of the isolates at species level **(Joung and Cote, 2002)**, 16SrDNA sequencing for the description of phylogenetic relationships **(Ash *et al*., 1992**) and pulse field gel electrophoresis (PFGE) for the differentiation of isolates at strain level **(Liu and Chen, 1997; Mendo *et al*., 2000**). Identification and succession of microorganisms involved in fermentation of Lima bean to produce *daddawa* using molecular method is yet to be documented. The present study therefore employed molecular method to identify the predominant bacteria during natural fermentation of Lima bean to produce *daddawa*.

MATERIALS AND METHODS

Matured dried lima bean seeds were purchased at Ita-ogbolu, Ondo State, Nigeria. Calabashes, cooking pots, washing bowls and sample dishes were obtained from a local market in Ibadan, Nigeria. The media used were obtained from Oxoid (UK) and LAB M (UK). Primers and reagents for molecular analyses were obtained from Inqaba Biotechnology, South Africa.

Lima bean fermentation

Lima bean fermentation was carried out according to the method used by **Farinde *et al*. (2014)**. Lima bean seeds were roasted, dehulled and cooked for 40 min. The cooked beans were drained and poured while still warm into clean calabash lined with clean banana leaves, covered with banana leaves before covering with another calabash. One calabash was prepared for each fermentation stage. The calabashes with their content were placed in an incubator for fermentation to take place at 35 ^{o}C ± 2 ^{o}C for 72 h. Sampling of the Lima beans under fermentation was carried out at 24 h intervals in triplicates.

Microbial analysis

Total viable count (TVC) was determined using the method described by **Abiose *et al*. (1986)** and **Omafuvbe *et al*. (2000)**. Aliquot (1.0 ml) of appropriately diluted sample was plated in triplicates on nutrient agar (NA) plates. The plates were then incubated aerobically at 35 ^{o}C ± 2 ^{o}C for 24 h. Colonies were counted and expressed as colony forming unit per gram (cfu g^{-1}) of the sample. Representative colonies were streaked repeatedly to obtain pure isolates. Preliminary identification of the bacterial isolates was based on cultural characteristics, Gram staining reactions **(Harrigan and McCance, 1976; Harrigan, 1998)**.

Molecular identification of Bacterial isolate

The isolated bacteria were identified using the method described by **Adelekan and Nwadiuto (2012)**. The experiments were carried out at National Center for Genetic Resources and Biotechnology (NACGRAB) and International Institute of Tropical Agriculture (IITA), both in Ibadan, Nigeria. The method involves DNA extraction, PCR amplification and DNA sequencing.

DNA extraction and quality verification

DNA was extracted using DNA extraction kit. ZR Fungal/Bacterial DNA MiniPrep™ protocol (ZYMO Research) following manufacturer's instructions. Modified method of **Adelekan and Nwadiuto (2012)** was used. Lysing of bacterial cells to bring out the DNA was done using bashing beads and lysis solution supplied in the DNA extraction kit instead of the method of freezing-thawing cycles used by Adelekan and Nwadiuto (2012). The extracted DNA was verified for purity and quality using quantitative and qualitative methods. Quantitative method was carried out using Nano drop spectrophotometer. DNA sample (1µl) was loaded using micro pipette into the Nano drop spectrophotometer and the absorbances of the samples were read at 260 and 280 nanometer. The concentration of the extracted DNA and the ratio of the absorbance were automatically generated and displayed on the equipment. The concentration must not be less than 10 ng/µl **(Leninger, 1975; Biobank, 2004)**. The ratio of the absorbance is also important during Polymerase chain reaction (PCR). It must be between 1.8 and 2.2 for optimum DNA concentration of high purity **(Leninger, 1975; Brown, 1993; Biobank, 2004)**.
Qualitative method was carried out using agarose gel electrophoresis. Agarose (1%) was prepared in Trisboric EDTA buffer (TBE) and melted in a microwave and cooled to 50 ^{o}C. The molten agarose was stained with Gel Green (20 µl). Gel Green fluoresce under UV light. Stained agarose was poured into gel caster in which comb had been placed at one end. The gel was allowed to solidify. The solid gel was placed in electrophoresis tank containing TBE buffer and the comb was gently removed. DNA (1 µl) was mixed with loading dye (7 µl) and loaded into wells created by the comb. The electrophoresis was allowed to run at 130 volts for about 1hour 30 min during which DNA molecules migrated through the agarose gel in the buffer.

Photographing the gel

The gel was removed from the electrophoresis tank and placed under U.V light in a documentation unit and viewed using a protective eye glasses. A Polaroid camera was placed over the documentation box and the gel was photographed. Sharp bands indicated quality DNA.

Amplification of DNA by polymerase chain reaction

Specific region of 16SrRNA gene of isolated DNA was amplified by reacting the cell solution (template DNA) (1µl) with polymerase chain reaction (PCR) master mix (8 µl), primers (1 µl) and nuclease free water (6 µl) and running the reaction cycles in a PCR thermocycler machine (Lexus Gradient –Eppendorff AG). The primers used in this study were universal primers, synthesized commercially by Inqaba Biotechnology, South Africa. The pair of primer consists of forward primer F 27 having nucleotide sequence AGAGTTTGATC(A/C)TGGCTCAG and reverse primer R 1492 having nucleotide sequence TACGG(C/T)TACCTTGTTACGACTT. Control tube (PCR reaction mixture minus template DNA) was also set along with the reaction tubes. The reaction was allowed to run 35 cycles at conditions of 94 ^{o}C for 3 minutes (Initial Denaturation), 94 ^{o}C for 1 minute (Final Denaturation), 55 ^{o}C for 30 seconds (Annealing), 72 ^{o}C for 2 minute (Extension) and 72 ^{o}C for 4 minutes (Final extension).
All PCR products were verified using 1% agarose gel electrophoresis as previously described. The samples were loaded along with Themo- scientific Gene ruler/ ladder (1 kbp).

DNA sequencing and Identification of isolates

Big Dye Terminator Cycle sequencing kit protocol was used for sequencing the amplicons and the gel was run on 3130 X 1.16 capillaries genetic analyzer from Applied Biosystems. The resultant sequences were submitted to Data base (Genbank, Germany) of National center for biotechnology Information (NCBI), the sequences were given accession numbers. The sequences were then matched with existing data in Data base using the Basic local alignment search tool (BLAST) algorithm. BLAST allows alignment of search sequence to thousands of different sequences in the data base **(Altschul *et al*., 1997)**. Species which had less than 90 % sequence identity with known representative sequence in data base were classified as unknown species and sequences with more than 90 % sequence identity were classified as known species.

RESULTS AND DISCUSSION

Microbial Counts during Natural Fermentation of Lima Bean to Produce *Daddawa*

The result of the Total viable count of microorganisms during natural fermentation of Lima bean to produce *daddawa* is shown in Figure 1.Total viable count increased from 4.65 log cfu g^{-1} at 0 h to 7.15 log cfu g^{-1} at 24 h, reached its peak (logarithmic phase) at 48 h of fermentation (8.08 log cfu g^{-1}) after which the count dropped to 7.40 log cfu g^{-1} at 72 h of fermentation. Similar trend of an initial increase in total viable bacteria count between 0 and 48 h of fermentation and subsequent drop in the count at 72 h of fermentation during production of *daddawa* have been reported **(Abiose *et al*., 1986; Omafuvbe *et al.*, 2000; Omafuvbe *et al.*, 2002; Enujuigba *et al.* 2008; Fadahunsi and Olubunmi, 2010)**. Increase in the total count at initial fermentation phase is probably due to availability of nutrients in form of carbon and nitrogen sources.

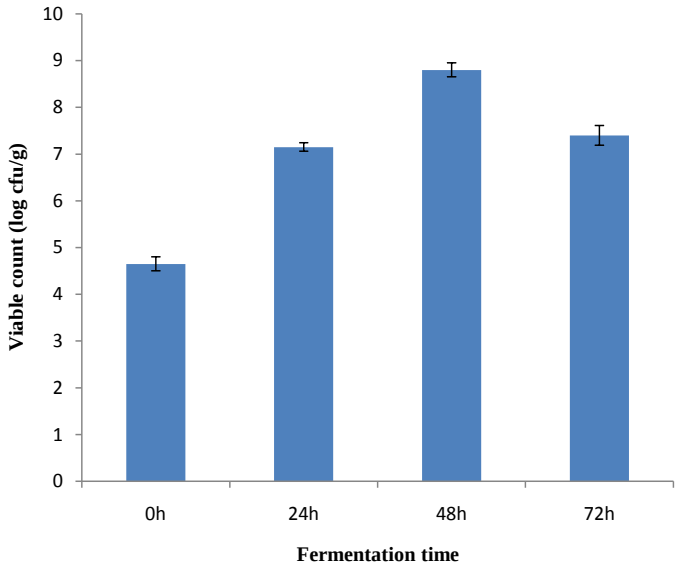

Figure 1 Total Viable Count of Microorganisms during Natural Fermentation of lima bean

Lima bean has been reported to have these nutrients in good quantities **(Yellavila *et al*., 2015)**. The drop in TVC after 48 h was probably due to depletion of nutrients and accumulation of metabolites. Among the likely metabolites of protein hydrolysis are short peptides and ammoniacal compounds which gave the product its desired flavor. These metabolites were likely more than the metabolites produced from hydrolysis of fat and carbohydrates present in the lima beans. The possible effect of these metabolites is the alkaline pH values which the microorganisms might possibly be able to tolerate up to 48 h of fermentation, after which some of the microorganisms may find the increased alkaline environment unfavorable for their growth and proliferation and so the total number of microorganisms declined.

Preliminary identification of bacterial isolates

Bacteria isolated from natural fermentation of lima bean to produce *daddawa* are shown in Table 1. The isolates were Gram positive, rods, catalase positive and spore formers. These characteristics allowed preliminary identification of *Bacillus* **(Harrigan and MacCance, 1976; Harrigan, 1998)**. Bacterial isolates 3, 9, 19 and 23 had rhizoid shape, isolates 7,13,16,18 and 20 had circular shape while the rest of the bacterial isolates (1, 2, 4, 5, 6, 8, 10, 11, 12, 14, 15, 17, 21, 22,24, 25 and 26) had irregular shape on agar plate. Similar preliminary phenotypic identification of bacterial isolates from *iru*, *afitirin* and *sonru* as belonging to genus *Bacillus* was reported by **Azokpota *et al.* (2007)**. This preliminary identification which showed the bacterial isolates as *Bacillus* was used to search for the appropriate primers for the molecular identification of bacteria isolates.

Table 1 Preliminary identification of Bacterial Isolates from Natural Fermentation of Lima Bean to Produce *Daddawa*

Isolate number	Appearance on agar plate	Shape	Gram's reaction	Catalase reaction	Presence of spore
1	Irregular	Rod	+ve	+ve	+ ve
2	Irregular	Rod	+ve	+ve	+ ve
3	Rhizoid	Rod	+ve	+ve	+ve
4	Irregular	Rod	+ve	+ve	+ ve
5	Irregular	Rod	+ve	+ve	+ ve
6	Irregular	Rod	+ve	+ve	+ ve
7	circular	Rod	+ve	+ve	+ ve
8	Irregular	Rod	+ve	+ve	+ ve
9	Rhizoid	Rod	+ve	+ve	+ ve
10	Irregular	Rod	+ve	+ve	+ ve
11	Irregular	Rod	+ve	+ve	+ ve
12	Irregular	Rod	+ve	+ve	+ ve
13	Circular	Rod	+ve	+ve	+ ve
14	Irregular	Rod	+ve	+ve	+ ve
15	Circular	Rod	+ve	+ve	+ ve
16	Circular	Rod	+ve	+ve	+ ve
17	Irregular	Rod	+ve	+ve	+ ve
18	Circular	Rod	+ve	+ve	+ve
19	Rhizoid	Rod	+ve	+ve	+ ve
20	Circular	Rod	+ve	+ve	+ ve
21	Irregular	Rod	+ve	+ve	+ ve
22	Irregular	Rod	+ve	+ve	+ ve
23	Rhizoid	Rod	+ve	+ve	+ ve
24	Circular	Rod	+ve	+ve	+ ve
25	Irregular	Rod	+ve	+ve	+ ve
26	Irregular	Rod	+ve	+ve	+ ve

Sequencing and BLAST result

Forward primer 27F and reverse primer 1492R were used to amplify the 16SrRNA gene of each of the bacterial isolates which resulted in well defined amplicons. When the amplicons were sequenced, the base sequences ranged from 911 in isolate number 8 to 1199 base sequence in isolate number 9 (Table 2).
When the sequences were matched with already existing similar sequences in Gene Bank Data base, all the isolates have their identity between 94 and 98% as shown in Table 2. Spore forming *Bacillus* species were identified as the major bacteria present during the fermentation process. Four isolates (isolates 12, 13, 15 and 24) did not match any identity. The identified isolates fell into 5 species of *Bacillus* (Table 3).These include *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus pumilus, Bacillus cereus and Bacillus anthracis.*
From the BLAST result, *Bacillus subtilis* was identified as the most predominant species during the fermentation of lima bean to produce *daddawa* (Table 3). **Azokpota *et al.* (2007)**, who used ITS – PCR – RFLP analysis to identify the bacteria isolated from three *daddawa* products (*iru*, *afitirin* and *sonru*), reported that *Bacillus subtilis* group represented the dominant species in the three condiments (*iru*, *afitirin* and *sonru*). **Adewunmi *et al*. (2013)** also reported that the result of polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) identified *Bacillus subtilis* as the consistent bacterial species associated with fermentation of *iru.* Previous studies by several authors using culture dependent methods and biochemical characterization have also reported *Bacillus subtilis* as being predominant during fermentation of beans to produce

Table 2 Result of the 16SrRNA Squence Analysis of the Bacterial Isolate

Isolate	Identity	(%) Similarity	Base sequence	Accession Number
1	*B. amyloliquefaciens* Strain FGYM6	98	1173	JN999853.1
2	*B. subtilis* Strain ov	98	1196	GU585579.1
3	*Bacillus sp.* Strain OS 1	98	1181	EF428970.1
4	*B. anthracis* Strain R5-331	97	1120	JQ65973.1
5	*B. anthracis* Strain yxc1-1	97	1050	JF701962.1
6	*B. subtilis* Strain C3	95	1179	JX120508.1
7	*B. cereus* Strain 25	95	1089	DQ42176.1
8	*B. subtilis* Strain 30L1-2	94	911	JN366795.1
9	*Bacillus sp*. Strain EGY-WCP9	95	1199	KF5623361.1
10	*B. subtilis* Strain BG-B7	97	1164	EU869248
11	*B. anthracis* Strain R5-331	97	1193	JQ659732.1
14	*B. pumilus* Strain DL-006	98	1151	KJ608548.1
16	*B. cereus* Strain VIT-AVJ	97	1174	KJ437489.1
17	*B. amyloliqueficiens*	97	1147	KC492052
18	B. *cereus* Strain HC23	97	1175	KJ206081.1
19	*Bacillus sp.* Strain HB38	96	1191	KF8638337.1
20	*B. cereus* Strain 400	98	1145	DQ420187.1
21	*B. amyloliquefaciens* Strain D.18	98	1146	AB813716.1
22	*B. amyloliquefaciens* Strain NK3-1	98	1158	HQ831391.1
23	*Bacillus. sp*. Strain HB38	97	1171	KF863837.1
25	*B. anthracis* Strain R5331	94	1154	JQ659732.1
26	*B. subtilis* Strain AB30	97	1142	JX188065.1

B - Bacillus

condiments **(Antai and Ibrahim, 1986; Odunfa and Oyewole, 1986; N'dir *et al*., 1994; Omafuvbe *et al*., 2000; Omafuvbe *et al*., 2002; Ouoba *et al*., 2004; Okpara *et al*., 2013)**. *Bacillus species* have been reported to be associated with fermentation of vegetable proteins **(Abiose *et al*., 1986: Kiers *et al*., 2000; Omafuvbe *et al*., 2002; Enujiugha, 2009; Ojinaka and Ojimelukwe, 2013)**. Inability to identify 15 % of the total isolates could probably be linked with some inadequacies in the nucleotide sequence in the genes of the representative chromosomes with which the primers could bind. **Azokpota *et al.* (2007)** also

reported that 16 % of isolates from *iru* and *sonru* (*daddawa*-like) could not be identified with molecular method.

Bacillus subtilis, *Bacillus amyloliquefaciens*, *Bacillus pumilus* were found to occur throughout the fermentation period (Table 4). The occurrence of *Bacillus subtilis* throughout the fermentation period and its predominance in fermenting beans has been reported by various authors **(Ogueke and Ariatu, 2004; Achi, 2005; Omafuvbe, 2008; Adelekan and Nwadiuto, 2012)**. *Bacillus subtilis* was identified as the best starter culture for fermentation of soybean to produce *daddawa* **(Omafuvbe *et al*., 2002)**. Of particular interest is *Bacillus amyloliquefacens* which also occur

Table 3 *Species* of *Bacillus* identified by 16SrRNA Sequence Analysis Using Basic Local Alignment Search Tool (BLAST)

Isolate number	% similarity	Bacterial *specie*
1, 17, 21, 22	98, 98, 98 and 98 respectively	*Bacillus amyloliquefaciens*
2, 6, 8, 10, 26	98, 94, 94, 97 and 97 respectively	*Bacillus subtilis*
3, 9, 19 and 23	98, 95, 96 and 97 respectively	*Bacillus species*
14	98	*Bacillus pumilus*
7, 16, 18, 20	95, 97, 97 and 98 respectively	*Bacillus cereus*
4, 5, 11, 25	97, 97, 97 and 94 respectively	*Bacillus anthracis*
12,13,15 and 24	No matched identity	

Table 4 Bacterial Succession during Natural Fermentation of Lima Bean to Produce *Daddawa*

Bacteria	Fermentation time (h)			
	0	24	48	72
Bacillus subtilis	+	+	+	+
B. amyloliquefaciens	+	+	+	+
B. pumilus	+	+	+	+
B. cereus	+	+	-	-
B. anthracis	+	+	-	-

+ = present - = not present

through-out the fermentation process. *Bacillus amyloliquefaciens* is a plant associated bacterium which is known for its ability to promote host plant growth through production of stimulating compounds and suppression of soil borne pathogens by synthesizing antibacterial and antifungal metabolites **(Niazi *et al*., 2014)**. Of concern is the occurrence *Bacillus cereus* and *Bacillus anthracis* in the fermentation process as their presence in the fermented product could pose public health problems **(Adelekan and Nwadiuto, 2012)**. Occurrence of these organisms did not exceed 24 h of fermentation, an indication that they are not important in the fermentation of lima bean. **Adelekan and Nwadiuto (2012)** similarly reported occurrence of *Bacillus cereus* and *Bacillus anthracis* during the first 48 h of fermentation of locust bean to produce *daddawa*. *Bacillus cereus* and *Bacillus anthracis* have been reported to be members of cultivable bacteria associated with fermented beans **(Choma and Granum, 2002; Ouoba *et al*., 2004; Oguntoyinbo *et al*., 2010)**.

The occurrence of *Bacillus cereus* and *Bacillus anthracis* or any other pathogen in the early stages of fermentation process could probably have arisen from handling of the beans after boiling and their inability to occur at the later stages of fermentation might be due to unfavorable environment for their survival **(Omafuvbe *et al*., 1999)** coupled with the of presence of *B. amyloliquefaciens* which might probably suppress or inhibit growth of pathogens. However, food borne diseases have not been reported in areas where these condiments (*iru/daddawa, afitirin, soniru)* are generally consumed **(Azokpota *et al*., 2007)**. **Azokpota *et al*. (2007)** thus suggested that further medical investigation have to be carried out to confirm whether *Bacillus cereus* strains identified in fermented condiments such as *iru/daddawa* are actually toxigenic.

There were no fungi isolated in the Lima bean fermentation process. This might be as a result of heat process prior to fermentation and probably an uncondusive environment for their growth and multiplication. Absence of fungi in the fermented Lima beans makes the product safe for consumption in terms of mycotoxin production **(*Abiose et al*., 1986)**.

CONCLUSION

The study concluded that molecular method could be used to identify the important microorganisms in fermentation of lima bean to produce *daddawa*. The method revealed that *Bacillus subtilis*, *B. pumilus* and *B.amyloliquefaciens* were majorly involved in the fermentation process with *B. subtilis* been predominant and that these three species of *Bacillus* could be tested and purified for use as potential starter for producing quality lima bean *daddawa*.

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