Induced Fermentation for the Production of Virgin Coconut Oil (VCO) Using *Bacillus cereus* Strain ST06 (MH475925.1) and *Bacillus velezensis* Strain Bv1-4 (OQ787546.1)

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Abstract: The use of bacteria in fermentations to produce functional foods has greatly increased in recent years due to the numerous associated benefits. Moreover, the increased demand for the production of virgin coconut oil (VCO) at household and industrial level as well as the need to overcome the problem of contamination associated with spontaneous fermentation justifies this research. Matured Coconut fruits were selected from which coconut milk used for the research was manually extracted using blender. Bacteria were isolated from spontaneously fermented coconut milk and steeped maize using standard technique. The isolates were characterized and identified using laboratory and molecular techniques. The isolates were further used as starter culture to carry out induced fermentation of coconut milk at laboratory conditions for 24, 48 and 72 hours for the production of VCO. During fermentation, the oil yield was determined using standard method. The molecular characterization confirmed the isolates were *Bacillus cereus* strain ST06 (MH475925.1) and *Bacillus valezensis* strain Bv1-4 (OQ787546.1). The two bacterial isolates were able to produce VCO from the coconut milk, although oil yield decreases as the duration of fermentation increases. In conclusion, the two (isolates) were found to possess the potential for producing VCO from coconut milk.

Key word: *Bacillus valezensis*, *Bacillus cereus*, Virgin coconut oil, Coconut milk, Induced fermentation

INTRODUCTION

Activities of microorganisms and/or their enzymes on food materials results in biological transformations that break down complex molecules into simpler ones. Thus, new food and food with better organoleptic property are ensured (Sharma *et al*., 2020). Fermentation for food production has been an age long process that dated back to the time of the earliest man. Fermented foods are globally accepted and widely consumed as local or exotic dishes in many communities, especially in the developing countries of the world. Many indigenous foods are processed through microbial fermentation in a non-specific manner either through spontaneous fermentation or back slopping. Each locality has its own preferred fermented food and adoptable methods of preparation. Benefits of fermented foods are enormous and cannot be over-emphasized. Notable importance of fermented foods spans through nutrient improvement and bioavailability, therapeutic values and economic boosting (Leech *et al*., 2020).

The involvement of bacteria and their edible metabolites in the food processing industries have experience a surge in the recent. This is due to the accrued advantages and the important roles of naturally processed foods in combating malnutrition and food related health challenges (Sharma *et al*., 2020).

Virgin coconut oil (VCO) is naturally derived from the milk from coconut (*Cocos nucifera* L,) fruit, of the Arecaceae (Palmae) family. The oil is obtained through microbial fermentation or mechanically by physical method. The processing methods may be variously modified to yield oil with desirable characteristics of edible oil (Djannah *et al*., 2022). Processing organisms and methods used are the major factors that define the nutritional and physicochemical properties of VCO (Angeles-Agdeppa *et al*., 2021). Virgin coconut oil produced through microbial fermentations has several advantages over other methods (Maini and Lopez, 2022). Such benefits include long shelf life, better aroma, and resistance to rancidity, more economical and highly conservative in terms of waste product generation (Maini and Lopez, 2022). Microbes for VCO processing are usually those with ability to produce the enzymes proteases, amylases and lipases which are necessary for the fermentation of the coconut milk. Such organisms are members of the Lactic acid bacteria (LAB), of which strains of *Lactobacillus plantarum* and *Lactobacillus casei* are the most commonly used (Olateru *et al*., 2020). Producing VCO using the lactic acid bacteria fermentation yields oil with better saponification and peroxide value, lower free fatty acid content and are more effective as antimicrobial (Alyaqoubi *et al*., 2015; Acharya *et al*., 2020; Benítez-Chao *et al*., 2021).

Applications of VCO have been severally reported globally and its merits over other types of oils have been variously documented. It is majorly accepted as edible oil and utilize in food processing, drug production, aesthetics and cosmetology, in the health sector as antibacterial, antiviral, antihelminthes, antiprotozoal, antioxidant and antifungal agent (Alyaqoubi *et al*., 2015; Bolyen *et al*., 2019; Acharya *et al*., 2020; Benítez-Chao *et al*., 2021). The coconut plant has numerous relevance to human life; highly nutritional with many useful byproducts (Pandiselvam *et al*., 2022). The high oil content stands it out in production of edible oil (Widari *et al*., 2021).

Despite the merits of VCO and its various applications, production is still mostly done at household levels using spontaneous fermentation. This process yields oil below standard and are prone to contamination. However, there are records of induced fermentation for VCO production (Adekola *et al.,* 2021), organisms for induced production has also been narrow, and mostly restricted to the homolactics. There is still paucity of knowledge about processing of VCO with other fermenters in the heterolactics. The increased demand for the production of VCO at household and industrial level, the demand for more organisms for production and the challenge of contamination during spontaneous fermentation justifies this research.

MATERIALS AND METHODS

Sample collection: Twenty (20) matured Coconut fruits were purchased at Ipata Market, Ilorin Metropolis, Kwara State, Nigeria and were transported immediately to the laboratory for further analysis within 4 hours.

Coconut milk extraction: The coconuts were manually dehusked and the shell broken. The solid endosperm was collected and the testa removed using sterile kitchen peeler. The endosperm (coconut balls) was disintegrated into thin pieces and blended with sterile distilled water in ratio 1:2 for 10 minutes. Blended mass was manually filtered for coconut milk extraction. The process was repeated twice and the volume of coconut milk obtained was determined and recorded. The extract of milk obtained was used for fermentation process (Adekola *et al*., 2021).

Isolation of associated bacteria during fermentation of milk extract: Associated bacteria were isolated from spontaneously fermented coconut milk and (steeped) grain respectively using MRS de-Man Rogosa and Sharpe (MRS) agar. Pour plate techniques was employed. Incubation was at 37^0C under for 48 hours. Two distinct colonies were sub-cultured repeatedly to get pure cultures. The pure cultures were labeled as “A” and “B”. The pure cultures were maintained on agar slants at 4^0C and for further analysis (Tagesu, 2018).

Identification of isolated bacteria: Gram staining for the determination of cell wall morphology (Tagesu, 2018), biochemical tests (indole, Methyl Red-Voges-Proskauer (MR-VP), citrate utilization, catalase, urease, oxidase and sugar fermentation) as well as molecular analysis were used to identify the isolated bacteria (Tagesu, 2018; Chandra *et al*., 2020; CLSI, 2022).

Molecular characterization of bacterial isolates: The genomic DNA of the bacterial isolates was extracted from the cultures using the Quick-DNATM Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target region was amplified using oneTaq(R) Quick load (R) 2X Master Mix (NEB, Catalogue No. M0486) with the primers as presented in the Table 1. The PCR products were run on a gel and cleaned up enzymatically using the EXOSAP method. The extracted fragments were sequenced in the forward and reverse direction (Nimagen, Brilliant Dye (TM) Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA sequencing Clean-up Kit(TM), Catalogue No. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific) for each reaction for every sample. BioEdit Sequence Alignment Editor version 7.2.5 was used to analyse the ab1 files generated by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI) (Atschul *et al*., 1997).

Induced fermentation for virgin coconut oil (VCO) production: Overnight (24 hours old) culture of the isolates were adjusted to 0.5 % McFarland standard (approximately equivalently to about 1.5 x 10^8 CFU/ml). The coconut milk in nine (9) jars was autoclaved at 121^0C for 15 minutes for sterilization. In groups of three sets, the first and second set of the coconut milk was inoculated with 10 ml (1.25 %) of isolate A and B respectively, while the third set was inoculated with 10 ml of 1:1 v/v of isolates A and B. The jars were labeled appropriately and incubated for 24, 48 and 72 hours for oil production (Adekola *et al.,* 2021).

Oil recovery: At the end of 24, 48 and 72 hours respectively, 100 ml of n-Hexane was added to the fermented mixture and allowed to stay for 24 hours to extract the oil. Oil separation was done using separating funnel. The oil collected was further purified to remove the n-Hexane through evaporation in water bath preset at 50^0C (Jasman *et al*., 2021). The yield of the oil in each stage was calculated as: %Yield = (mass of oil sample used/mass of oil obtained) ×100.

RESULTS

The isolated bacteria were Gram positive rods, citrate, catalase, oxidase positive but negative to urease test and were identified as *Bacillus* spp (Table 2). Evidences from the (16s) ribosomal subunit of the RNA identified the isolates as *Bacillus velezensis* Bv 1-4 (OQ787546.1) and *Bacillus cereus* ST06 (MH475925.1) based on their genetic sequences with percentage ID similarity of 91.79 and of 99.20 % (Plate 1, Figure 1 and Table 3). There was sequential decrease in the oil yield as the duration of fermentation increases in the three sets. The oil yield by *Bacillus cereus* (MH475925.1) treatment (8.89 and 3.47 %) was comparatively higher than the yield from *Bacillus valezensis* (OQ787546.1) (7.32 and 2.87 %) at 24 and 72 hours respectively. The oil yield from by the bacteria was similar at 48 hours (2.36 and 2.43%). The use of the two organisms for VCO production did not have any profound effect on oil yield. All the treatments experienced lower yield at 72 hours (Table 4).

Table 1: Deoxyribonucleic acid primer

Name of Primer	Target	Sequence 5’ to 3’
16S-27F	16SrDNA sequence	AGAGTTTGATCMTGGCTCAG
16S-1492R	16SrDNA sequence	CGGTTACCTTGTTACGACTT

Table 2: Biochemical characteristics of isolated bacteria from fermented coconut milk

Characteristics	Isolate A	Isolate B
Oxidase	-	+
Catalase	-	+
Urease	-	-
Citrate	-	+
Indole	-	-
Coagulase	-	-
Glucose	-	-
Lactose	-	-
Sucrose	+	-
Voges Proskaur	+	-
Methyl Red	+	-
Gram’s reaction	+ rod	+ rod
Probable Organisms	*Clostridium* spp	*Bacillus* spp

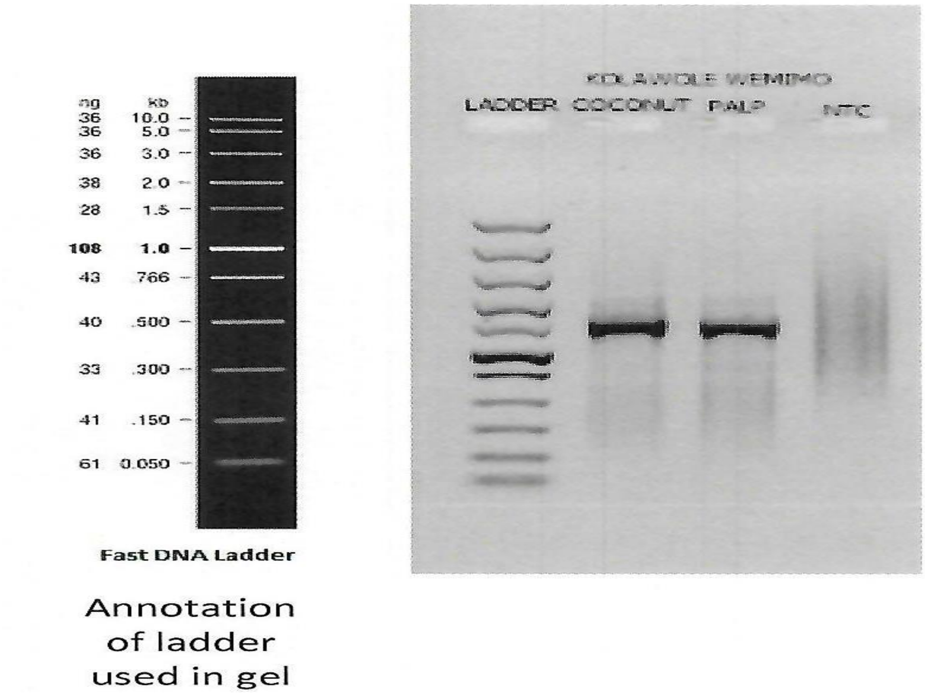

Plate 1: Agarose gel indicating the 16s target region
Key: Isolate A (from fermented coconut milk); Isolate B (from fermented grain)

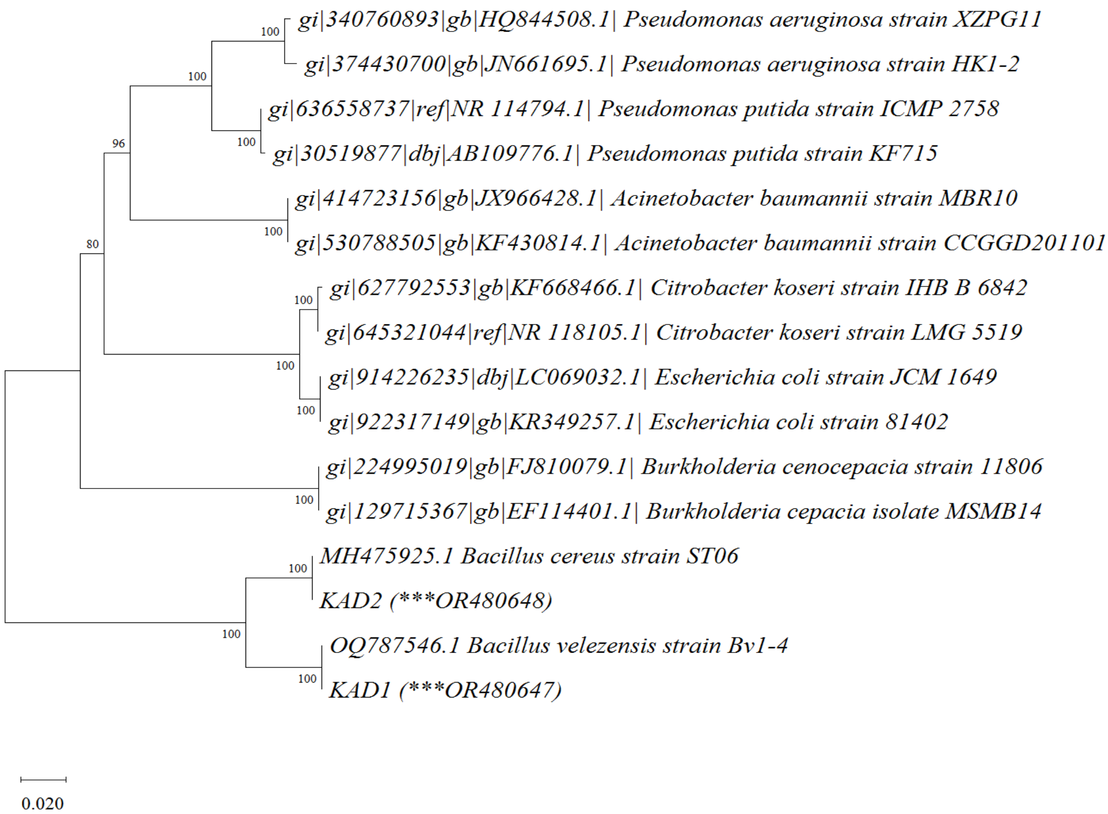

Figure 1: Phylogenetic tree of relationship of *Bacillus cereus* and *Bacillus velezensis* strains isolated based on 16S rRNA constructed by neighbor joined method with other *Bacillus*

Table 3: Molecular identities of the isolated bacteria

Sample	Percentage ID	Confirmed Organisms	GenBank Accession
Isolate A	91.79 %	*Bacillus velezensis*	OQ787546.1
Isolate B	99.20 %	*Bacillus cereus*	MH475925.1

Table 4: Percentage oil yield of samples during the period (hours) of fermentation

Sample	Incubation period (hours)	Oil yield (%)
Bacillus cereus	24	8.89
Bacillus velezensis		7.32
B. cereus and velezensis		8.66
Bacillus cereus	48	2.36
Bacillus velezensis		2.43
B. cereus and velezensis		1.94
Bacillus cereus	72	3.47
Bacillus velezensis		2.87
B. cereus and velezensis		1.90

DISCUSSION

There is an undeniable urgency to discover new organisms for induced fermentation for the production of VCO, develop cost effective procedure and overcome the menace of bacterial contamination in the effort to increase the quantity and availability of edible oil for household and industrial use. This would in no small way, combat the problem of health related diseases associated with other sources of edible oil and it would also enhance the small and middle scale local/indigenous industries engaged in its production. The economy of the developing nation would equally be positively affected. Microbial fermentation remains one of the most viable methods of producing VCO from coconut milk for both domestic and industrial consumption. Therefore, the present status of VCO locally produced is grossly inadequate to meet the domestic demand for the product for direct consumption needless of industrial use. In order to scale up production, novel organisms are major determinant.

The method explored in this study diverged from the previous conventional means of microbial fermentation using the popularly known *Lactobacillus plantarum,* a homolactic bacterium (Olateru *et al*., 2020; Ng *et al*., 2021) by examining other organisms that can also carry out fermentation for VCO production. The synergistic effect of two bacteria on oil yield was also examined. Two *Bacillus* bacteria, identified as *Bacillus velezensis* Bv1-4 (OQ787546.1) and *Bacillus cereus* ST06 (MH475925.1) were established respectively as being able to produce oil, via fermentation, of coconut milk respectively. The genus *Bacillus* have been reported to contain species and strains can produce extracellular enzymes that hydrolyze carbohydrates, proteins, and lipids hence, are capable of carrying out fermentation. Non-pathogenic *Bacillus* species with potential for food fermentation includes *amyloliquefaciens, licheniformis, megaterium, acidoterrestris, coagulans* and *subtilis* (Lorenzo *et al*, 2018). Although *B. subtilis* can become an opportunistic pathogen under certain conditions and majority of the common species are food spoilage agents (Lorenzo *et al*, 2018).

The percentage oil yield obtained from the induced fermentation using the isolated *Bacillus valezensis* Bv1-4 (OQ787546.1) and *Bacillus cereus* ST06 (MH475925.1) or both was however, comparatively lower than those reportedly obtained from fermentation involving *Lactobacillus plantarum* by previous authors (Olateru *et al*., 2020; Adekola *et al*., 2021). The difference in yield could be as a result of the sources and types of coconut fruit used, laboratory conditions and the organisms used. The use of *Bacillus valezensis* Bv1-4 (OQ787546.1) and *Bacillus cereus* (MH475925.1) as starter culture for induced fermentation yielded very small percentage oil, the organisms probably have antagonistic effect on oil production rather than the expected synergism. Previous works on VCO have engaged singular culture (Olateru *et al*., 2020; Adekola *et al*., 2021) hence, the necessity of assessing the effect of using two cultures on oil yield.

Incubation period does not increase oil yield during this study rather the oil yield dropped tremendously after 24 hours of incubation. Most fermentation for VCO production has been within 24 to 48 hours (Olateju *et al.*, 2020). This observation could be traceable to the fact that bacteria are more active at their exponential phase of growth and activities reduce beyond this point due to nutrient depletion, oxygen tension and production of toxic secondary metabolites.

CONCLUSION

The use of *Bacillus valezensis* Bv1-4 (OQ787546.1) and *Bacillus cereus* ST06 (MH475925.1) isolated and used as starter culture was examined. The *Bacillus valezensis* Bv1-4 (OQ787546.1) and *Bacillus cereus* ST06 (MH475925.1) were found to be able to produce VCO from coconut milk via fermentation, thereby diverging from previous conventional method of using the *Lactobacillus plantarum* and other members of the homolactics.

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