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Biochemical Characterization of Microbial Isolates and Effect of Concentration and Incubation Time of Plant and Cow Milk Yoghurt using Response Surface Methodology

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The objective of the research was to evaluate the biochemical characteristics of the various microbial isolates used in the production of plant based yoghurt, the optimal growth temperatures and the effect of incubation time and inoculum concentration on the taste and desirability of the

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yoghurt. Pure cultures of five (5) microbial strains (*Lactobacillus acidophilus*; *Lactobacillus bulgaricus*; *Lactobacillus casei*; *Lactobacillus lactis*; *Streptococcus thermophilus*) were obtained from commercial yoghurt and evaluated for their biochemical characteristics, bacterial growth temperatures and synersis. The isolated organisms were used to ferment plant-based milk product produced from blends of tigernut, Dates and Coconut. Response Surface Methodology (Face centered composite design) was used to obtain an optimized ratio formulation of 0.167 for coconut, 0.667 fortigernut and 0.167 for dates. Bacterial growth temperatures were evaluated at 4°C, 27°C, 37°C,40°C, 45°C and 60°C respectively. Results showed that full bacterial growth was noticed at temperatures of 40-45°C. The taste of the yoghurt improved from 6.58 to 6.71 when the incubation temperature changed from about 45°C to 44°C and the duration of incubation changed from 15.4 hrs to 14.7 hrs.

Keywords: Yoghurt; inoculum concentration; tigernut; dates; coconut.

1. INTRODUCTION

Production of yoghurt has been carried out over the years using bacterial cultures such as Lactobacillus *bulgaricus* and Streptococcus *thermophilus*. The fermentation process involved in Yoghurt production leads to a decrease in pH and the development of enhanced organoleptic properties [1]. Dairy milk has been the primary source of yoghurt product, however there is an increasing need of plant-based yoghurt products due to the health benefits associated with them. Makinen [2] reported that plantbased yoghurts are generally made from fermenting aqueous extracts obtained from different plant raw materials such as legumes, Cereals and Oil seeds. The use of Plant based alternatives can be adduced to the high cost of dairy based products and the presence of α-lactalbulin and β-lactoglobulin which have been shown to cause food allergy in some individuals [3]. The problem of lactose intolerance, a condition caused by the inability of the individual to digest lactose has become a factor limiting the use of dairy milk and its product [4]. The consumption of probiotic yoghurts has been shown to have functional benefits to the consumers. Probiotics have been reported to play a role in reducing the occurrence, duration and severity of flu [5].Various investigations have been carried out on the possibility of using Plant based milk sources such as Coconut, Soybean and Tigernut as an alternative to dairy milk [6,7,8]. Milk extracted from tiger nut, apart from being nutritious, has been recommended for persons that do not tolerate gluten or are allergic to cow milk and its derivatives [9].

Tigernut milk is very popular in Nigeria. The milk is a viscous cream coloured liquid with a very short shelf-life (48 h) when produced traditionally [10]. It has been reported to be of great health benefits, It is rich in mineral content and aids bones, tissue repair, muscles, the bloodstream and body development [11]. Other health benefits are the prevention of colon cancer, coronary heart diseases, obesity, diabetes and gastro-intestinal diseases [12,13]. Tigernut has a total carbohydrate content of 47.9 – 75.88% of which starch is 17.2-39.2% and imparts undesirable sensory attributes such as a chalky mouthfeel to the products [14].

Coconut milk can play a vital role in balancing the nutritional deficiency of our diet. Coconut (Cocos*nucifera*L.) is the most extensively grown and used nut in the world, which belongs to the palm family (*Arecaceae*). Besides, it is an important commercial crop in many tropical countries, contributing significantly to their economies [15]. The micro-organism used mainly in yoghurt production are Lactic acid bacteria which are utilized in the fermentation process. *Lactobacillus* have been shown to have an inhibitory role on pathogenic bacteria [16].

The goal of this research was to evaluate the biochemical characteristics of the various microbial isolates, the optimal growth temperatures and the effect of the incubation time and inoculum concentration on the taste and desirability of the yoghurt.

2. MATERIALS AND METHODS

2.1 Preparation of Yoghurt Starter Culture

The morphology and type of bacterial colonies were investigated via serial dilution and cell count experiments on MRS agar. Gram staining and microscopic examination of the growth indicated that 5 distinct colonies designated ANOL1-5 wereobserved on MRS agar plates from triplicate plates (Table 2). Morphological characteristics revealed that the isolates were predominantly smooth margined with raised elevation, gram positive and white to creamish-white in colour.

2.2 Morphological and Biochemical Identification of Bacterial Isolates

Three commercial yoghurts (Fan, Farmfresh and Habib) were obtained, serial diluted and grown on different media. Visually distinctive colonies were randomly selected and purified by repeat streaking on anti-fungal supplemented MRS agar, Nutrient agar and Muller Hinton agar. Colonies that grew on both agars were gram stained to check deviations from the standard in accordance with the standard Gram staining procedure described by Tortora [17]. Biochemical tests were carried out in accordance with standard methods described by Cheesbrough [18]. Distinct isolates were subjected to morphological identification aided by Gram staining with the following parameters: Colour, Shape, Elevation, Surface and Biochemical characterization were performed according to known methods [19].

2.3 Acclimatization of the Bacterial Culture to Ferment the Plantbased Milk

The biochemically confirmed pure isolates were engineered to produce the plantbased yoghurt. The optimized milk blend with the ratio 0.167coconut, 0.667tigernut, 0.167dates was also used to achieve the desired product. The bacteria isolates were pressured to ferment 100% of the plant base milk by preparing a fortified blend of plantbased milk (tiger nut, dates, and coconut) with cow milk at various ratios of 1:10 to 10:1. The batch container having the highest volume of plant milk and the evidence of microbial activities for yoghurt formation were serially diluted. The diluent 10^{-6} and 10^{-7} after serial dilution were pour plated and anaerobically incubated in sterile MRS agar, distinct colony was sub cultured and later grown in sterilized MRS broth, the broth was centrifuged at 10,000rpm and harvested into sterile water. The pure culture was then used as inoculum. Incubation temperature, inoculum concentration and incubation time were used as process variables and all varied as provided in the experimental design (Table 1) in order to produce various samples of yoghurt formulation.

2.4 Sensory Evaluation of the Probiotic Yoghurt from the Optimized Milk Blend

Sensory evaluation of prepared **yo**ghurt blends was carried out in accordance with the method described by Everitt [20]. The 9-point hedonic rating test was used for the assessment, ranging from 1- dislike to 9- like extremely (9-Like Extremely, 8-Like Very Much, 7-Like Moderately, 6-Like Slightly, 5-Neither Like nor Dislike, 4-Dislike Slightly, 3-Dislike Moderately, 2-Dislike Very Much, 1-Dislike Extremely). The sensory qualities assessed were colour, sweetness, taste, aroma, appearance and overall acceptability.

Table 1. Design key with the code and actual values of the independent variable

Independent Variable		1	0	+1
A.	Inoculum concentration (%)	0.05	1.02	2.0
B.	Incubation duration (h)	14.0	16.0	18.0
C.	Incubation temperature (^oC)	37	41	45

Table 2. Culture and morphological characteristics of *Lactobacillus* strains obtained on MRS agar

Microbial Code	Colour	Gram Reaction	Elevation	Margin	Surface	Microscopy observation
ANOL 1	White	Positive	Raised	Smooth	Spreading	Rod with rounded clusters
ANOL 2	Cream	Positive	Raised	Smooth	Spreading	Rod, rounded, in chain
ANOL 3	White	Positive	Raised	Smooth	Spreading	Cocci, single, in pair
ANOL 4	Cream	Positive	Raised	Smooth	Spreading	Rod, single pair, chain
ANOL 5	Cream	Positive	Raised	Smooth	Small beads	Spherical chain

Key: ANOL 1: Lactobacillus acidophilus; ANOL 2: Lactobacillus bulgaricus; ANOL 3: Lactobacillus casei; ANOL 4: Lactobacillus lactis; ANOL 5: Streptococcus thermophilus

2.5 Experimental Design

Response surface methodology (Face centered composite design) was used to obtain an optimal ratio formulation of coconut, tigernut and dates extracts used in the production of yoghurt. The model generated is quadratic and significant ($p<0.05$). Other terms in the ANOVA that exhibited significant p-values include A (inoculum concentration), BC (interaction of incubation time and temperature), B^2 (square of incubation time and C^2 (square of incubation temperature). The P-value for lack of fit is not significant and $AdjR^2$ value (0.7567 or 75.67%) is high. Being a Response Surface Methodology (RSM) design, it would first assume a Quadratic polynomial model as presented in the equation below

$$Y = \beta_0 + \sum^3_{i=1} \beta_i X_i + + \sum^3_{i=1} \beta_{ii} X_i^2 + \sum^3_{i=1} + \sum^3_{j=i+1} \beta_i X_i X_j$$

Where Y is the response, β_0 constant, β_i the linear coefficient, β_{ii} the quadratic coefficient, $\beta_{ij}$the interaction coefficient, X_i and X_j are the independent variables. The model for taste was therefore fitted based on the terms that showed significant p-values from the analysis of variance. The taste of yoghurt was modeled as specified below bore the regression coefficient values for the terms:

$$Taste = \beta_0 + A + BC + BC^2 + C^2$$

$$Taste = 6.80 - 0.16A - 0.12BC + 0.23B^2 - 0.39C^2$$

3. RESULTS

3.1 Biochemical Characterization of the Isolates

Biochemical characterization used to confirm the type of organisms isolated, revealed that all isolates were citrate, coagulase, indole, oxidase, urease, methyl red, nitrate, arginine, galactose, mannitol and raffinose negative. However, sugar tests conducted showed lactose, glucose, maltose, fructose and sucrose were positive. This means the organisms can utilize these sugars as carbon sources for metabolism. Our plant yoghurt contains sucrose and it is important to note that these isolates can thrive in the sample. Salt and Acid tolerance on the isolates shows they can survive in low pH and hence in yoghurt. One attribute of LABs as probiotics is their ability to survive in salt environment and this test confirmed further the species. The results are displayed in Table 3.

3.2 Bacteria Temperature Growth in Milk Profile

The isolates were exposed to various temperatures to evaluate survival. At a low temperature of 4 -10°C, no growth was seen. At 27°C, there was partial growth for all isolates.Growth was observed at 37°C. At 40°C and 45°C, Full growth was observed while no growth was observed at high temperature of 60°C. Most *Lactobacillus* exhibits thermophilic tendencies and all isolates thrived most at temperatures between 40°C and 45°C as shown in Table 4.

Fig. 1 showed the interaction effect of inoculum concentration and incubation duration on the taste of the probiotic yoghurt. From the plot, the interaction between duration and inoculum concentration shows that the taste of the yoghurt improved from 7.1 to 7.2 when duration changed from 17hrs to 17.5hrs and inoculum concentration changed from about 0.7% to 0.4%.

Fig. 2 shows the contour plot on taste when there is an interaction between incubation temperature and inoculum concentration. When the temperature changed from 44.5°C to 43.5°C and inoculum concentration changed from 1.02% to 0.45%, taste improved from 6.51 to 6.64.

Fig. 3 shows the interaction between the Incubation temperature and the incubation duration on the taste of the probiotic yoghurt. From the contour, the taste of the yoghurt improved from 6.58 to 6.71 when the incubation temperature changed from about 45°C to 44°C and duration of incubation changed from 15.4hrs to 14.7hrs. There were 15 solutions that gave desirability of 1.0. Desirability ranges between 0.1 to 1.0, and the higher the desirability value the more the predicted value of the response variable gets closer to its actual (experimental) value. As shown in Fig. 3, desirability increased from 0.379 to 0.804 when inoculum concentration changed from 2.0% to 0.54% and time changed from 14.3hrs to 15hrs. Incubation temperature was kept at 45°C.

Results of Syneresis are shown in Table 5.The syneresis result of the cow yoghurt (CY) and plant yoghurt (PY) over a 21 days period at different times (30, 60 and 90 minutes) showed that at 30 mins, cow milk yoghurt had values reducing from 12.85 to 10.00, while plant yoghurt reduced from 16.60 in day one to 15.56 at day twenty-one. At 60 mins interval, cow milk yoghurt

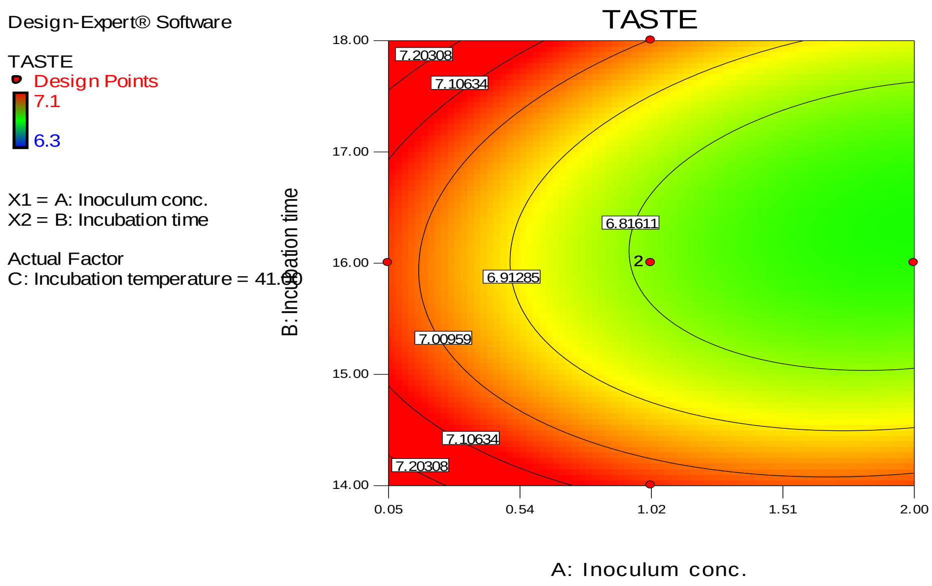

Fig. 1. Inoculum concentration and incubation time

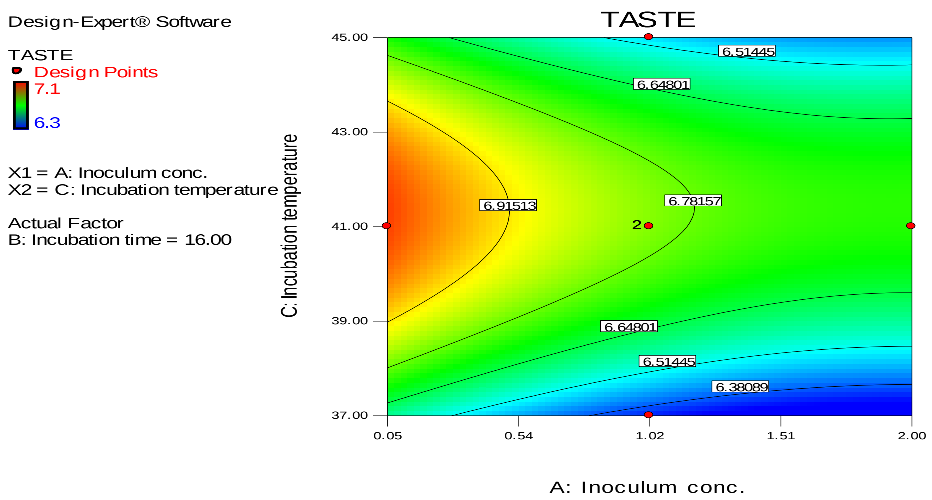

Fig. 2. Contour plot for Inoculum conc and Incubation temp

reduced from 16.60 in day one to 15.56 at day twenty-one, while plant yoghurt decreased from 20.00 to 17.00 respectively. At 90 mins, cow milk yoghurt syneresis reduced from 18.00 to 14.95, while plant yoghurt reduced from 24.75 to 21.05 for day one and day twenty-one respectively.

Result also showed that syneresis of the different yoghurt samples increased under a given storage condition as the time increased. Cow milk yoghurt stored at day one increased with time from 12.85 in 30mins to 18.00 after 90mins. Syneresis was seen to decrease throughout the storage period. Syneresis which is also termed "Wheying off", was more in plant yoghurt than in cow yoghurt.

4. DISCUSSION

The combination of tigernuts, coconuts and date fruits can be said to have increased the aesthetic and sensory value of the yoghurt produced from the blend and this is in agreement with previous report [21]. The colours of samples 3 (0.167 coconut, 0.667tigernut, 0.167 dates), 6 (0.000 coconut, 1.000 tigernut, 0.000 dates) and sample 8 (0.000 coconut, 0.000 tigernut , 1.000 dates) were liked very much by the panelists while all other samples were liked moderately.

Table 3. Biochemical characterization of microorganisms towards starter culture development

Biochemical test	**ANOL 1**	**ANOL 2**	**ANOL 3**	**ANOL 4**	**ANOL 5**
Citrate	-	-	-	-	-
Coagulase	-	-	-	-	-
Indole	-	-	-	-	-
Oxidase	-	-	-	-	-
Urease	-	-	-	-	-
Methyl –Red (MR) Test	-	-	-	-	-
Motility test	-	-	-	-	-
Salt endurance	1-9%	1 – 10 %	1-5%	1-10%	1-9%
Acid endurance	-	>2	-	-	>2
Nitrate reduction	-	-	-	-	-
Casein decomposition	-	-	-	-	-
Arginine producing ammonia	-	-	-	-	-
Glucose gas production test	-	-	-	-	-
Glucose	+	+	+	+	+
Galactose	-	-	-	-	-
Mannitol	-	-	-	-	-
Lactose	+	+	+	+	+
Maltose	+	+	+	+	+
Raffinose	-	-	+	-	+
Fructose	+	+	+	+	+
Sucrose	+	+	+	+	+
Trehalose	-	+	+	-	+
Organism	*Lactobacillus acidophilus*	*Lactobacillus bulgaricus*	*Lactobacillus casei*	*Lactobacillus lactis*	*Streptococcus thermophilus*

Key: - Negative + Positive

ANOL 1: Lactobacillus acidophilus; ANOL 2: Lactobacillus bulgaricus; ANOL 3: Lactobacillus casei; ANOL 4: Lactobacillus lactis; ANOL 5: Streptococcus thermophilus

Table 4. Bacteria temperature growth in milk from tigernuts, coconuts and dates

Isolates	Temperature					
	4°C	27°C	37°C	40°C	45°C	60°C
Lactobacillus bulgaricus	NG	PG	G	FG	FG	NG
Lactobacillus acidophilus	NG	PG	G	FG	FG	NG
Streptococcus thermophiles	NG	PG	G	FG	FG	NG
Lactobacillus lactis	NG	PG	G	FG	FG	NG
Lactobacillus casei	NG	PG	G	FG	FG	NG

Key: No Growth (NG) Growth (G) Partial Growth (PG) Full Growth (FG)

Table 5. Syneresis (%) of the plant and cow yogurt

Time Minutes	Sample	Strong Period Days			
		1	7	14	21
30	CY	12.85	12.00	10.75	10.00
	PY	16.60	16.60	16.60	15.65
60	CY	15.80	15.00	13.00	12.65
	PY	20.00	18.45	18.00	17.00
90	CY	18.00	17.85	15.50	14.95
	PY	24.75	23.45	23.00	21.05

CY- Cow yoghurt; PY- Plant yoghurt

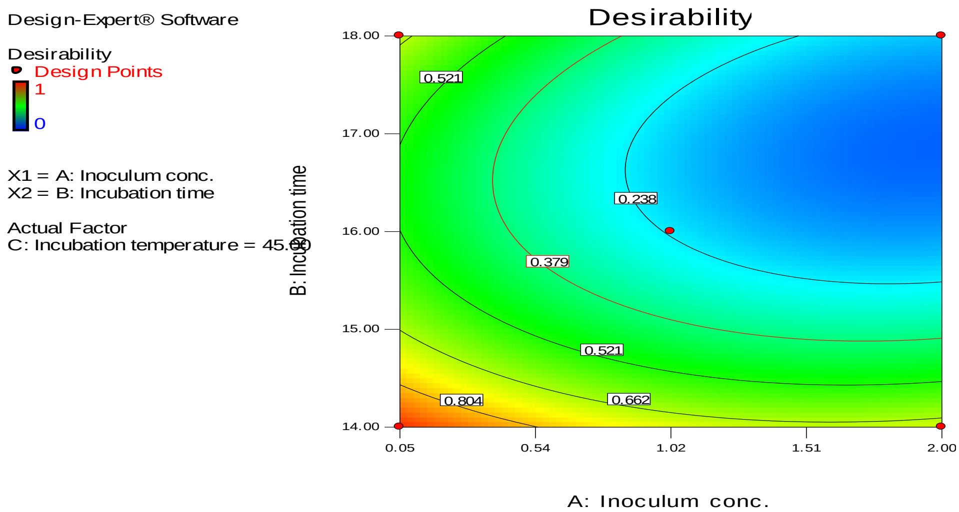

Fig. 3. Contour plot for desirability of taste

In this study, Taste and Overall acceptability were significantly different (P<0.05) in their sensory scores. This is in agreement with findings from other studies, which revealed that incubation duration, incubation temperature, and starter culture concentration are factors to consider in plant-based yoghurt production [22, 23]. 2% inoculum as well as incubation temperature of about 41°C yielded the most acceptable (8.65 in sample 8) yoghurt as scored by the panelists. Lower concentrations of inoculum (0.05%) produced yoghurts that were slightly liked regardless of temperature and time.The process variables (Inoculum concentration, Incubation time and Incubation temperature) did not have and significant difference in the colour, sweetness, aroma and appearance of the sixteen (16) yoghurt samples. Whey separation (syneresis), also generally known as "wheying off" is a problem of fermented milks during storage and can be explained as the oozing off of liquid from the surface of a gel [24]. It negatively affects consumer perception and makes them believe the milk is microbiologically deteriorating. This study revealed thatsyneresis was higher in the plant yoghurt than the animal yoghurt. A number of factors influence yoghurt's viscosity, namely, milk composition, heat treatment, standardization method, selection of microbial culture, inoculum concentration, temperature, and fermentation duration [25]. The viscosity trend seen in yoghurt can be described as the interaction of the fiber and protein with the water of yoghurt, this forms a strong network that increases the resistance of yoghurt to flow. Stirred yoghurt has a peculiar characteristic, different from set yoghurt as its three-dimensional gel matrix is no longer visible. Stirred yoghurt has a weak gel system comprising of weakly associated clusters of proteins that make up the network. During storage, stirred yoghurt exhibits thixotropic behavior and the viscosity increased in both the plant yoghurt and the dairy yoghurt. In this study, the viscosity increased significantly (P>0.05) and syneresis reduced during storage. This behavior is contrary to what is seen in set yoghurt, where viscosity decreases with storage. This can be attributed to increased lactic acid production as storage progressed, resulting in a more stable get structure [26]. The use of stabilizers, such as, pectin, gelatin and starch, to try to prevent wheying-off is strongly recommended, although another common approach is to increase the total solids content of yoghurt milk.

5. CONCLUSION

This study revealed that incubation duration, incubation temperature, and starter culture concentration are factors to consider in plant-based yoghurt production. The viscosity trend seen in yoghurt can be described as the interaction of the fiber and protein with the water of yoghurt, this forms a strong network that increases the resistance of yoghurt to flow.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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