British Microbiology Research Journal
4(2): 224-234, 2014

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Sourcing Starter Cultures for *Parkia biglobosa* Fermentation Part II: Potential of *Bacillus subtilis* Strains

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Authors' contributions

This work was carried out in collaboration between all authors. Author EYA initiated the research, managed the literature searches, isolated and prepared the working cultures, performed the starter-culture fermentations, determined the pH, degree of hydrolysis, ammonia nitrogen, performed statistical analysis of data; prepared the extracts for enzymes assays; and wrote the first draft of the Manuscript. Author WV designed the study, coordinated the group study (Head of Group). Author BS wrote the protocol, performed the enzymes' assays. Author KY wrote the protocol and performed the haemolytic tests on isolates. Author DJ prepared the Questionnaire and coordinated the sensory evaluation of the starter-culture fermented 'iru' products and performed the statistical analysis on the data. All authors read and approved the final manuscript.

Original Research Article

Received 16th August 2013
Accepted 24th October 2013
Published 8th November 2013

ABSTRACT

Aim: To select good strains of *Bacillus subtilis* for use as starter culture in the fermentation of *Parkia biglobosa*.
Study Design: Fifteen (15) strains of *Bacillus subtilis* group obtained from commercial samples were used in starter-culture fermentation of *Parkia biglobosa* seeds to produce 'iru'.
Place and Duration of Study: Food Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC), Pahumthani, Thailand, between March to May 2010.

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Methodology: The quality of the starter culture-fermented products were compared on the bases of sensory evaluation, degree of hydrolysis (DH), level of ammonia nitrogen (NH_3-N), pH and enzymatic activities. The 15 strains were also screened for haemolytic activity.
Results: On the basis of the sensory scores of 5 parameters (color, odor, consistency, texture and over-all liking), particularly the over-all liking, 5 strains were rated the best (in descending order): BC4333 > 8B > 2B > 7A > 5A, amongst the 15 tested. There were good correlations between pH and DH (r= 0.926), DH and NH_3-N (r=0.962) and between pH and NH_3-N (r=0.945). The strain BC4333 produced the very soft variant of 'iru' ('iru-pete'), without the addition of 'kuuru' (local potash). The quantity of extracellular enzymes (protease, amylase, pectinase, phytase and lipase) produced during fermentation varied significantly. None of the 5 strains was haemolytic on sheep blood agar.
Conclusion: The 5 strains of *Bacillus subtilis* (BC4333, 8B, 2B, 7A, 5A) that showed potentials of being used as starter cultures for industrial production of 'iru', were non-hemolytic on blood agar.

Keywords: Bacillus; Parkia biglobosa; starter-culture; haemolytic; enzymes.

1. INTRODUCTION

African locust bean (*Parkia biglobosa*) is a highly valued multipurpose tree. The roasted seeds are used as a coffee substitute known as 'Sudan coffee' or 'café nègre'. The mealy pulp from the fruits is eaten or is mixed with water to make a sweet and refreshing drink rich in carbohydrates; the boiled pods are used to dye pottery black, while the ash is applied as a mordant [1]. 'Iru' is a bacterial-fermented product of the seeds, consumed in many West African countries, as both soup condiment and meat substitute. It is known by different names in West Africa; these include 'iru' (among the Yoruba ethnic groups), 'dawadawa' (among the Hausa ethnic groups), 'soumbala' (in French-speaking countries), 'kpalagu' (in Ghana), 'kinda' (in Sierra Leone) and 'netetou' (in Gambia) [2]. The mixed bacterial populations involved in the spontaneous fermentation include strains of *Bacillus subtilis* group, *Staphylococcus epidermidis* and *Lactobacillus* species [3]. Quality of 'iru' varies, with respect to taste, texture, color, odor (level of ammonia) and stringiness of the product. Factors that influence quality of product include the quality of the seeds, the expertise of the producer and the strain(s) of *Bacillus* involved. Previous reports have shown that the strains vary in growth rate and extracellular enzymes' production in broth cultures [4]. The proteinases produced by the strains of *Bacillus* included serine, neutral and an esterase [5].

In order to enhance the fermentation process, and have standardized 'iru' products, with improved keeping qualities, there is need to obtain few good strains of *Bacillus subtilis* group, which can be used as starter cultures [6]. Thus the objective of this work was to screen some *Bacillus subtilis* group isolates, through starter culture experiments; and evolve a few strains which have the potentials of being developed into starter cultures for industrial scale production of 'iru'.

2. MATERIALS AND METHODS

2.1 Preparation of Inocula and Substrate for Starter Culture Fermentations

Fifteen (15) strains of *Bacillus subtilis* group, previously isolated from commercial samples of 'iru' (strains 8B, 1A 9B, 10B, 2B, 7A, 3A, 4A, 6A, 3B, 9A, 5A, 6C) and 'thua-nao' (strains

BC4333 & BC7123) were used in the starter culture fermentations [7]. The cultures were maintained (at -70ºC) as stock cultures at Food Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand. Each organism was grown overnight in 5-ml sterile Nutrient Broth (NB) in test tube at 35ºC, 200rpm; then used to inoculate 50ml NB in 250ml flasks, and incubated for 18h. Culture was centrifuged (Eppendorf Centrifuge 5810R, Model Rotor F-34-6-38) at 10,621g (4ºC) for 10min; and cells' pellets were re-suspended in 5ml sterile distilled water. Cells' density was determined by spectrophotometric reading of suitably diluted culture at 600nm. An $OD_{(600nm)}$ of 1.0 was equivalent to 10^7cells/ml.

Dried seeds were soaked in water (ratio 1: 5 w/v) for 2h, autoclaved at 121^{o}C for 15min and dehulled manually. Clean cotyledons in distilled water (1:2 w/v) were re- sterilized (121ºC for 15min) and drained of excess water. Cotyledons (300g) were inoculated with 1ml of suitably diluted culture to give final concentration of 10^4cells/g, mixed and transferred aseptically into sterile fermentation containers (perforated stainless steel bowls, lined with aluminium foil and muslin cloth). Each container was covered with aluminium foil and incubated at 35ºC for 36h.

2.2 Evaluation of Starter-culture Fermented Products

Sensory evaluation, pH, degree of hydrolysis (DH) and ammonia-nitrogen (NH_3-N) of the products were determined. Extracellular enzymes' activities in the fermented products were also quantified.

2.2.1 Sensory evaluation

Five grams (5g) each of the starter-culture fermented samples were assessed by trained panelists [8]. The parameters: consistency (i.e. viscosity of bacterial film on surface of cotyledons), texture, color, odor (level of detection of ammoniacal odor) and over-all liking were scored on a 9-point Hedonic scale; where 9 and 1 were 'like extremely' and 'dislike extremely' respectively.

2.2.2 pH

One gram (1g) of sample was ground to form a pulp, using a clean mortar and pestle. Five milliliters (5ml) of distilled water was added to make slurry. The pH meter (Mettler Toledo) was standardized with buffer solutions (pH 4.0 and 9.0).

2.2.3 Degree of hydrolysis (DH)

The method of Benjakul and Morrissey [9] was adopted. The sample for $\boldsymbol{L_{max}}$ was a mixture of 0.5ml of sample and 4.5ml of 6M HCl. Hydrolysis was run at 100^{o}C for 24h. Standard curve was prepared, using stock solution of 3mM L-leucine.

Calculation: $DH\ (\%) = [(L_t-L_0)/ (L_{max}-L_0)] \times 100$

where L_t corresponded to the amount of α-amino acids released at 36h in fermented samples. L_0 was the amount of α-amino acids in unfermented sample and L_{max} was the total free amino group after acid hydrolysis (6 M HCl at 100^{o}C for 24 h).

2.2.4 Determination of ammonia-nitrogen

The ammonia-nitrogen (NH_3-N) in samples was determined by distillation (Gerhardt Rapid Distillation System Type VAP 20). Known weight of ground sample (1g) was added into 3g of magnesium oxide (MgO) in a distillation flask. Hundred milliliters (100ml) of water was added and the mixture was distilled under the following conditions: Program 1 (0 sec), Program 2 (0 sec), Program 3 (600sec), and Program 4 (50%). The column was washed in-between samples; varying programs 3 and 4 to 300 sec and 75% respectively. The distillate was collected in 25ml boric acid containing methyl-red and bromocresol green indicator; and titrated against 0.1N HCl. The quantity of NH_3-N in samples was calculated thus:

$$NH_3\text{-N (mg/100g)} = (0.1*V_{HCl})/1000*(17.007/W_s)*100$$

Where W_s is the weight of sample to the fourth decimal point, and V_{HCl} is the titre volume of HCl.

2.3 Enzyme (Quantitative) Assays

2.3.1 Extraction method

The crude enzyme was prepared by adding 40 ml of distilled water to 10 g of 'iru' and mixed by stomacher at 200 rpm for 3 min. The suspension was centrifuged at 10,621g (4ºC) for 10 min; supernatant was collected for enzyme assay.

2.3.2 Amylase

The amylase was assayed by following the method of Rick and Stegbauer [10]. The reaction mixture contained 0.1 ml of enzyme sample, 0.2 ml of (1%) soluble starch in 0.1 M citrate-0.2 M phosphate buffer (pH 7.2). The mixture was incubated at 35ºC for 10 min. The reaction was terminated and color developed by DNS method [11]. One unit of amylase activity (U) was the amount of enzyme that will release 1µg glucose per minute.

2.3.3 Lipase

Lipase assay was by following the Dharmsthiti et al. [12] method. The reaction mixture contained 0.1 ml of enzyme sample, 0.9 ml of reaction reagent (1 part of solution A (0.62% (w/v) of 4-nitrophenylpalmitate in 2-propanol) and 9 parts of solution B (0.1% (w/v) gum arabic in 50 mM Tris-HCl pH 7.2). The reaction reagent was prepared freshly before use. The reaction was carried out at 35ºC for 30 min. The reaction was terminated by adding 0.2 ml of 10% (w/v) SDS. The color developed was determined by spectrophotometer at 410 nm. One unit of lipase activity (U) was defined as the amount of enzyme that will release 1µg of 4-nitrophenol per minute.

2.3.4 Pectinase

Pectinase was assayed following the method of Mandels et al. [13]. The reaction mixture contained 0.1 ml of enzyme and 0.2 ml of (1% citrus) pectin in 0.1 M citrate-0.2 M phosphate buffer, pH 7.2. The mixture was incubated at 35ºC for 30 min. The reaction was terminated and color developed by DNS method. One unit of pectinase activity (U) was the amount of enzyme that will release 1µg of glucose per minute.

2.3.5 Phytase

Phytase was assayed by the method of Engelen et al. [14]. The reaction mixture contained 0.5 ml of enzyme and 1 ml of (8.4g/l) sodium phytate in 0.2 M acetate buffer, pH 7.2. The reaction mixture was incubated at 35ºC for 60 min and stopped by adding 1 ml of freshly prepared color reagent. One unit of phytase activity (U) was the amount of enzyme that will release 1µg of inorganic phosphate per minute.

2.3.6 Protease

The procedure of An et al. [15] was adopted for protease assay. The reaction mixture contained 0.1 ml of enzyme sample and 1 ml of (1 %) sodium caseinate in 0.1 M citrate-0.2 M phosphate buffer, pH 7.2. The reaction was incubated at 35ºC for 30 min. The reaction was terminated by addition of 0.2 ml of 50% (w/w) TCA. One unit of protease activity (U) was the amount of enzyme that will release 1µg of tyrosine per minute.

2.4 Screening of Isolates of Choice (from Starter Culture Experiment) for Haemolytic Activity

Toxigenicity of the choice isolates of *Bacillus subtilis* were determined by screening them for haemolytic activity on blood agar. One loop-full of 18h cultures grown in tryptic soy broth (TSB) were streaked on sheep blood agar plates. The ability of cultures to induce haemolysis was determined after incubating plates for 24h at 37ºC.

2.5 Statistical Analysis

Data obtained from sensory evaluation, pH, DH, ammonia nitrogen and enzymatic activities in the starter-culture fermented products were subjected to statistical analysis (ANOVA and Duncan's) in SPSS 17.0 package

3. RESULTS AND DISCUSSION

3.1 Sensory evaluation of fermented products

The result of sensory evaluation of the starter-culture fermented samples is shown in Table 1 Based on the scores for the 5 parameters, particularly the over-all liking, the *Bacillus* strains BC4333, 8B, 2B, 7A, and 5A were rated the best five (in descending order), amongst the 15 isolates tested. Though colonization of the surface of the cotyledons by the bacteria to form a creamy/whitish film was not copious on the product fermented by *Bacillus* strain BC4333, it was significantly softer in texture (by pressing between fingers) than the other products. Odunfa and Adewuyi [16] had reported that the quality of 'iru' was affected by the strain of *Bacillus* species involved in the fermentation. The local people add local potash, called 'kuuru' to produce the soft variant of 'iru' [17]. Results of this research have shown that 'iru-pete', the soft variant of 'iru' (which is preferred by some people), can be produced without addition of 'kuuru'.

Table 1. Sensory scores of starter-culture fermented 'iru' products.

Isolate/ Sample code	Scores (**) for sensory parameters of 'iru'				
	Consistency	Texture	Color	Odor	Over-all liking
* 8B	7.6±.548^{a}	7.8±.837^{ab}	7.8±1.304^{a}	7.2±.837^{a}	7.4±.894^{ab}
1A	3.4±1.14^{cd}	4.2±1.095^{de}	7.2±.837^{a}	2.4±.548^{c}	3.8±1.923^{ef}
9B	2.0±1.225^{d}	5.0±2.00^{cde}	6.6±1.14^{a}	2.6±1.342^{bc}	3.0±1.0^{f}
10B	3.2±2.387^{cd}	5.0±.707^{cde}	6.6±.547^{a}	3.8±1.788^{bc}	4.8±.836^{cdef}
* 2B	7.4±2.073^{ab}	5.4±1.14^{cde}	7.0±.707^{a}	5.4±2.074^{ab}	7.0±1.0^{abc}
* 7A	7.4±.894^{ab}	6.2±1.304^{bcde}	7.6±1.14^{a}	5.4±2.41^{ab}	6.4±1.342^{abcd}
3A	3.2±.49^{cd}	6.4±.548^{bcd}	7.0±.707^{a}	4.2±1.304^{bc}	4.8±1.923^{cdef}
4A	3.6±2.70^{cd}	4.2±1.788^{de}	6.4±.894^{a}	3.8±2.49^{bc}	4.2±2.387^{def}
6A	2.4±1.516^{cd}	4.0±2.00^{e}	6.2±1.30^{a}	3.8±1.92^{bc}	4.0±2.0^{def}
3B	3.4±2.51^{cd}	4.6±2.074^{cde}	6.2±1.48^{a}	3.4±2.191bc	3.6±1.816^{ef}
9A	4.0±2.345^{cd}	4.0±0.212^{e}	5.4±1.14^{a}	5.0±2.236^{abc}	5.0±2.0^{cdef}
* 5A	7.4±.894^{ab}	6.6±1.516^{abc}	6.6±1.52^{a}	4.8±2.049^{abc}	5.6±1.673^{abcde}
6C	7.25±1.5^{ab}	6.0±1.414^{bcde}	7.0±1.41^{a}	4.7±2.217^{abc}	5.5±1.291^{abcde}
* BC4333	7.8±.836^{a}	8.6±0.548^{a}	7.4±1.342^{a}	7.0±1.581^{a}	7.8±.836^{a}
BC7123	5.0±2.739^{bc}	5.0±2.121^{cde}	5.8±2.28^{a}	7.0±1.871^{a}	5.2±2.28^{bcdef}

** The best five strains on basis of over-all liking.*

*** Hedonic scale of 1 (dislike extremely) to 9 (like extremely)*

Values are mean and standard deviation of five replicates.

Values that have the same superscript in same column are not significantly different at P = .05

3.2 Physicochemical Properties of Fermented Products

Potash reduces the nutritional quality of foods as it binds with the protein, rendering the latter unavailable for assimilation in the body.

The pH and DH of the unfermented cotyledons and 15 fermented samples is shown in Table 2. The coefficient of determination, R^2 was 0.811. The general trend observed was that as the DH increased, the pH increased; and the level of ammonia in products increased with increase in pH.

Table 3 shows the results of the haemolysis test of the choice isolates on sheep blood agar. Except *Bacillus cereus* C113, (which served as positive control), other organisms, including *Bacillus subtilis* BCC 4333 (negative control) and isolates tested (BC4333, 8B, 2B, 7A, and 5A) did not induce haemolysis. The non-haemolytic nature of the 5 choice strains (*Bacillus* strains BC4333, 8B, 2B, 7A, and 5A) confirms their suitability in being used as starter-cultures for industrial-scale production of 'iru'.

Ability to effect desirable physicochemical changes in the substrate is an important criterion in the choice of starter cultures. The parameter used to select *Bacillus* isolates (from 'soumbala') having potentials as starter culture for controlled fermentation of African locust bean was the ability to degrade non-digestible oligosaccharides. Two isolates of *B. subtilis* were reported to have shown strong ability to degrade the oligosaccharides [18]. Crude enzymes' production by the 5 *Bacillus* spp. isolates in the starter-culture fermented samples (at 35ºC for 36h) is shown in Fig. 1.

Table 2. The pH and DH (%) of starter-culture fermented 'iru' samples

Sample	pH and DH of starter culture fermented 'iru' samples	
	pH (±sd)	DH(±sd)(%)
Unfermented	6.69±.02[a]	0.00[a]
8B	6.88±.117[b]	16.697±0.809[b]
1A	8.10±.061[i]	47.832±4.676[hij]
9B	8.04±.012[hi]	50.179±0.753[j]
10B	7.93±.057[fg]	46.606±0.792[hi]
2B	7.82±.044[e]	49.735±3.521[ij]
7A	7.79±.047[e]	43.242±2.296[g]
3A	8.01±.01[gh]	47.049±4.619[hij]
4A	7.93±.04[fg]	45.511±1.217[gh]
6A	7.92±.021[f]	37.349±0.91[e]
3B	7.90±.052[f]	47.701±1.547[hij]
9A	7.620±.036[d]	33.125±2.371[d]
5A	8.047±.032[hi]	40.296±3.504[f]
6C	7.920±.02[f]	31.534±2.014[d]
BC4333	7.943±.006[fg]	33.881±3.025[d]
BC7123	7.267±.029[c]	26.136±1.651[c]

DH: Degree of hydrolysis; Values are mean and standard deviation of triplicate determinations; Values that have the same superscript in same column are not significantly different at P = .05.

Table 3. Result of haemolytic test of choice isolates on sheep blood agar

Organism	Haemolysis
1. *Bacillus cereus* C113 (Positive control)	β
2. *Bacillus subtilis* BCC 4333(Negative control)	γ
3. 8A	γ
4. 2B	γ
5. 7A	γ
6. 5A	γ
7. BC433	γ

***Remarks: Haemolysis** is the ability of test bacteria to break down red blood cells; **β-haemolysis** is a complete lysis of red blood cells (the agar under and around the colony appears lightened and transparent);*
***γ-haemolysis** means the cultures tested did not induce haemolysis (the agar under and around the colony was unchanged).*

3.3 Enzymes Assays

The *Bacillus* strain BC4333 gave the highest amylase and pectinase activities (2,173.1 and 303.8 U/g dry weight fermented sample, respectively); while no enzyme activity was in control (un-inoculated cotyledons). The very high amylase and pectinase activities exhibited by the *Bacillus* strain BC4333 could have contributed to the extremely soft texture (paste) of the fermented product; as the polysaccharides and pectin in the seeds would have been hydrolyzed by the two enzymes.

The *Bacillus* strains 5A and 7A showed the highest lipase (580.9 U/g dry weight fermented sample) and phytase (237.2 U/g dry weight fermented sample) activities respectively; while *Bacillus* strain 2B showed the highest activity of protease (125.8 U/ g dry weight fermented

sample). The variation in the quantity of extracellular enzymes produced by these 5 strains corroborates the earlier report of Aderibigbe and Odunfa [4], that *Bacillus* species involved in 'iru' production varied significantly in their physiological activities.

Fig. 2 shows the correlation chart between pH, NH_3-N and DH for the selected samples and the unfermented substrate. There were good correlations between pH and DH (R= 0.926), DH and NH_3-N (R=0.962) and between pH and NH_3-N (R=0.945). Azokpota et al. [19] reported that some volatile compounds released during natural fermentation of *Parkia biglobosa* to 'iru' included aldehydes, ketones, pyrazines, alcohols and esters.

An essential property that all starter cultures involved in food production must have is to be non-toxigenic. Ostensvik et al. [20] reported the presence of cytotoxic *Bacillus* spp belonging to the *B. cereus* and *B. subtilis* groups in Norwegian surface waters which was used in food processing. In a bid to bring about production of safe 'okpehe', a traditional fermented soup condiment through starter culture fermentation, Oguntoyinbo and Sanni [21] screened fifty strains of *Bacillus* species for their toxigenic potentials; the results showed that 40% of the isolates were toxigenic.

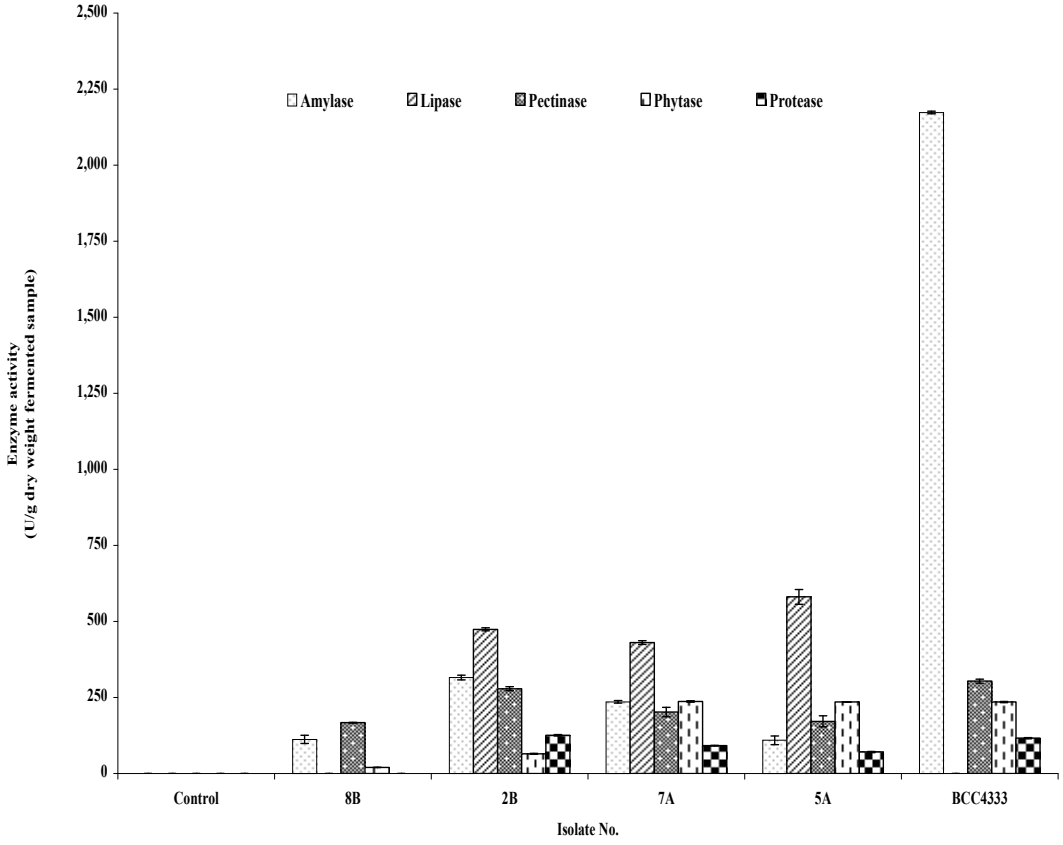

Fig. 1. Enzyme production by five selected strains of *Bacillus subtilis*

Legend: Bars indicate standard deviation from triplicate determinations. Activity was assayed at pH 7.2 and 35ºC.

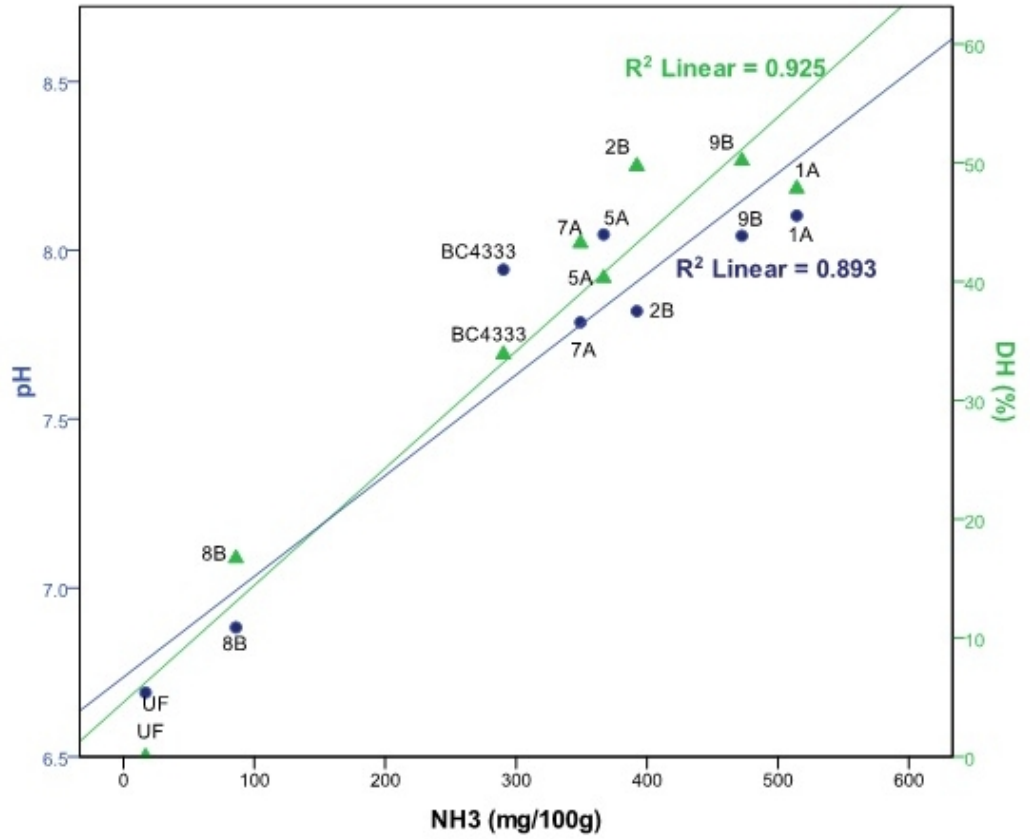

Fig. 2. Correlation chart between NH_3-N, DH and pH for selected starter-culture fermented samples

Legend: UF: Unfermented substrate, 1A, 2B, 5A, 7A, 8B, 9B and BC4333 are starter-culture fermented products. NH_3-N: ammonia nitrogen; DH: Degree of hydrolysis.

4. CONCLUSION

This study has elucidated 5 strains of *Bacillus subtilis* group, which have potentials for being used as starter cultures in industrial fermentation of 'iru'. The *Bacillus* strains are: BC4333, 8B, 2B, 7A, and 5A. One of the strains (BC4333), which was highly amylolytic and pectinolytic, produced very soft variety of 'iru' (;iru-pete'), without the addition of potash ('kuuru'). All the choice strains were non-haemolytic on blood agar.

ACKNOWLEDGMENTS

EYA wishes to acknowledge the TWAS-UNESCO Associateship Scheme and National Centre for Genetic Engineering and Biotechnology (BIOTEC), Thailand, for the financial support and opportunity to use the facilities at the Food Biotechnology Research Unit, BIOTEC.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Burkill HM. The useful plants of West Tropical Africa. 2nd ed. Vol. 3. Kew: Families J–L. Royal Botanic Gardens; 1995.
2. Odunfa SA, Dawadawa. In: Reddy NR, Pierson ND, Salunkhe DK. editors. Legume-Based Fermented Foods. Boca Raton: CRC Press; 1986.
3. Odunfa SA. Micro-organisms associated with fermentation of African locust bean (*Parkia filicoidea*) during iru preparation. Journal of Plant Foods. 1981;3:245-250.
4. Aderibigbe EY, Odunfa SA. Growth and extracellular enzyme production by strains of *Bacillus* species isolated from fermenting African locust bean, iru. Journal of Applied Bacteriology. 1990;69:662-671.
5. Aderibigbe EY, Schink B, Odunfa SA. Extracellular proteinases of *Bacillus* spp. isolated from fermented African locust bean, 'iru'. Food Microbiology. 1990;7*:*281–293.
6. Latunde-Dada GO. Fermented foods and cottage industries in Nigeria. Food Science; 1995. Accessed 23 September 2012. Available: http://www.unu.edu/unupress/food/V184e/ch3.htm.
7. Aderibigbe EY, Visessanguan W, Sumpavapol P, Kongtong K. Sourcing starter cultures for *Parkia biglobosa* fermentation I: Phylogenic grouping of *Bacillus* species from commercial 'iru' samples. International Journal of Biotechnology and Molecular Biology Research. 2011;2(7):121-127.
8. Larmond E. Laboratory Methods for Sensory Evaluation of Food. Editor 1st ed. Canada: Canada Publishing Centre; 1977.
9. Benjakul S, Morrissey MT Protein hydrolysates from Pacific whiting solid wastes. Journal of Agricultural and Food Chemistry. 1997*;*45:3423-3430.
10. Rick W, Stegbauer HP. α–amylase measurement of reducing groups. In: Bergmeyer HU. ed. Methods of Enzymatic Analysis. Weinheim:Verlag Chemie; 1974.
11. Miller G. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 1959;31:426-438.
12. Dharmsthiti S, Pratuangdejkul J, Threeagool G, Luchai S. Lipase activity and gene cloning of *Acinetobacter calcoacetiou* LP009. Journal of General and Applied Microbiology. 1998;44:139-145.
13. Mandels M, Andreotti R, Roche C. Measurements of saccharifying cellulase. Biotechnology Bioengineering Symposium. 1976;6:21-33.
14. Engelen AJ, Vanderheeft FC, Randsdorp PHG, Smit ELC. Simple and rapid determination of phytase activity. Journal of AOAC International. 1994;77:760-764.
15. An H, Seymour TA, Wu JW, Morrissey MT. Assay systems and characterization of Pacific whiting (*Merluccius productus*) protease. *Journal of Food Science,* 1994;59:277-281.
16. Odunfa SA, Adewuyi EY. Optimization of process conditions for the fermentation of African locust bean (*Parkia biglobosa*) II. Effect of starter cultures. Food Chemistry Microbiology Technology. 1985;9:118-122.
17. Odunfa SA. Biochemical changes in fermenting African locust bean (*Parkia biglobosa*) during iru fermentation. Journal of Food Technology. 1985*;*20:295-303.

18. Ouoba LII, Diawara B, Christensen T, Mikkelsen JD, Jakobsen M. Degradation of polysaccharide and non-digestible oligosaccharides by *B. subtilis* and *B. pumilus* isolated from Soumbala, a fermented African locust bean (*Parkia biglobosa*) food condiment. European Food Research and Technology. 2007;224(6):689-694.
19. Azokpota P, Hounhouigan JD, Annan NT, Nago MC, Jakobsen M. Diversity of volatile compounds of afitin, iru and sonru, three fermented food condiments from Benin. World Journal of Microbiology and Biotechnology. 2008;24(6):879-885.
20. Ostenvik O, From C, Heidenreich B, O'Sullivan K, Granum PE. Cytotoxic *Bacillus* spp. belonging to the *B. cereus* and *B. subtilis* groups in Norwegian surface waters. Journal of Applied Microbiology. 2004;96(6):903-1004.
21. Oguntoyinbo FA, Sanni AI. Determination of toxigenic potentials of *Bacillus* strains isolated from 'okpehe', a Nigerian fermented condiment. World Journal of Microbiology and Biotechnology. 2007;23(1):65-70.

Peer-review history:
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