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Protein enrichment of Irish potatoes by fermentation process using mutant isolates of *Latobacillus bulgaricus*

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ABSTRACT

The study undertook the mutational selection of lactic acid bacteria used for the fermentation of peeled *Solanum tuberosum* (Irish Potatoes, Subtrate) in order to isolate mutants that may produce fermented Irish potatoes product with improved nutritional quality (i.e increase protein content). Lactic acid bacteria were isolated from local yoghurt ("kindirmo" in hausa language) that were morphologically and culturally characterised and found to be predominantly *Lactobacillus bulgaricus*. Four (4) mutants selected randomly from *Lactobacillus bulgaricus* subjected to UV-light irradiation at 20 secs , 25 secs, 30 secs and 35 secs periods of exposure. The mutants exhibited similar morphological and cultural characteristics as the wild type strain. Fermentation of peeled Irish potatoes with wild type *Lactobacillus bulgaricus* gave no significant increase ($P>0.05$) in crude protein content from 7.37±0.42 g/100 g on the zero day to 8.73±0.43 g/100 g on the 5th day of fermentation, whereas, the mutant isolates gave a significant increase ($P<0.05$) of crude protein content from 7.37±0.42 g /100 g (zero day of fermentation) to 11.52±0.62 g/100 g, 12.18±0.62 g/100 g, 9.72±0.88 g/100 g and 14.50±0.52 g/100 g at 6th day of fermentation respectively. The mutant isolates, Lb35, showed the highest crude protein content (16.91±0.73 g/100 g) increase after the 5th day of fermentation of Irish Potatoes (*Solanum tuberosum*). The genetic analysis revealed that the *Lactobacillus bulgaricus* studied did not harbour any extrachromsomal element.

Keywords: Irish Potatoes, mutagenesis and selection, amino acids, plasmids, mutants, wildtype, fermentation.

INTRODUCTION

Biotechnology has been broadly defined as the utilisation of biologically derived molecules, structure, cells or organisms to carry out a specific process. This shows that the world of biology is undergoing a revolution of far-reaching implications, where microbes, plants and eukaryotic cells can be programmed to produce or overproduce normal or scarce natural products. The beauty of modern biotechnology lies in its specificity: one can target the production of an important flavour, colour or enzyme in many folds (Bruce, et al., 1988).

Irish Potatoes (*Solanum tuberosum*) is a perennial plant of the *Solanaceae* or nightshade family and is commonly grown for its starchy tuber; it is one of the world most widely grown tuber crop and the fourth largest

crop in terms of fresh produce (after rice, wheat and maize) (Hamilton et al., 2004). Because of the nutrient contents of plant foods, today's lifestyle related disease are best prevented through dietetic therapy (South African Potatoes, 2005). Albeit the Irish potatoes proteins are lacking in essential amino acids (SAP, 2005).

Axelsson (1998) and Adams and Moss (1995) stated that "lactic acid bacteria " are a group of gram positive bacteria, non-respiring, non-sporing end product of the fermentation of carbohydrates.

Fermentation has been defined as a dynamic process during which several catabolic and anabolic reactions proceed simultaneously depending on several conditions including substrate micro flora and environmental factors, which usually result in the breakdown of carbohydrates and other nutrients to give products such as Alcohols, Acids, Amino Acids, other metabolites and antibiotics including small amount of energy (FAO, 1998. Fermented foods play important role in providing food security, enhancing livelihoods and improving the nutrition and social wellbeing of millions of people around the world. It also leads to improved food preservation, increasing the range of raw materials that can be used to produce edible food products and removing anti-nutritional factors to make food safe to eat (FAO, 1998).

Mutagenesis is the process by which DNA is experimentally altered by physiochemical means to produce a stable change in the 'Gene'. Selection is the process of finding the mutant in the sea of a million "normal" bacteria (Elander, 1982; Roland, 1984). Mutagenesis and Selection have been used to produce bacteria which excrete high concentrations of amino acids (Shiio, 1982). Some of the mutagenic tools are Site-directed mutagenesis, direct selection, environmental stress, transposons, physical tools such as Ultraviolet light and chemical agents (Williams and Michael, 2000).

Mutation provides the basis of genetic studies (Williams and Michael, 2000). This study seeks to utilise one of the mutagenic techniques for improvement of *Lactobacillus spp* for fermentation of Irish potatoes with possible increase in protein (amino acids).

MATERIALS AND METHODS

Sample collection

Lactic acid bacteria strains (*Lactobacillus bulgaricus*) were isolated from "kindirmo" bought from Fulani women hawking it in University of Jos, Jos, Nigeria.

Isolation of lactic acid bacterial strains

Lactic acid bacterial strains were isolated from "kindirmo" culture on a selective medium (MRS agar) for lactic acid bacteria (Oxiod, 2004), and the strain identified according to its incubation temperature (30 °C) and growth in acidic medium. The strain was further characterised according to its morphological, cultural, physiochemical and biochemical characteristics using standard methods (refs) stock cultures were maintained at 4 ˚C on MRS medium for further use.

Fermentation process

The substrate was washed, peeled and mashed, 100 g of the mash substrate was placed into conical flask, mixed with 1ml of the isolated *Lactobacillus bulgaricus* and allowed to undergo fermentation under microaerophilic condition at 30 ^{o}C in oven for 7 days. On a daily basis, samplings of each of the 7 sets of fermented potatoes were taken to check for pH, dried matter and proximate analysis.

Induction of mutagenesis

Mutagenesis with ultra violet (UV) light were done according to Kamal et al. (2003).

UV – mutagenesis

Lactobacillus bulgaricus were grown at 30 °C in 100ml of tryptone soya broth (TSB) to cell optical density (O.D 600) of 0.2–0.3. The cells were harvested by centrifugation at 5,000 X g for 15min and washed twice in 100 ml cold, sterile, 0.9% NaCl solution. Portion of cell suspensions (8 ml aliquots) was transferred to sterile petri dishes and radiated with UV – light (254 nm) for four different periods (20, 25, 30 and 35 sec). Each irradiated sample was centrifuged at 5,000 X g for 15 min and re-suspended in 10 ml TSB and incubated at 30 °C for 18 hr. The cultures were then diluted serially into sterile 0.9% NaCl solution and 0.1 ml of serial dilution were plated on to MRS agar plates and incubated at 30 °C. Then mutants were isolated.

Characterisation of mutant isolates

Cell morphology was performed according to Ridge (1982), catalase test according to Schieri and Blazevic (1981), physiological test was as described by Oxoid (2004) and biochemical test was as according to the methods of Harrigan and McCance (1993), Sambrook et al. (1989) and Tserovska et al. (2002).

Isolation of plasmids

Plasmid analysis was carried out using the "Simple method for extracting plasmid DNA from lactic acid bacteria" by Frere (2008).

Statistical analysis

The statistical analysis used were simple standard deviation, percentage, ANOVA and correlation at 95% level of significance.

RESULTS

Isolation and identification of lactic acid bacteria

Microscopic examination of the isolates revealed rods-like cells that showed no catalase activity; motile, facultative anaerobic , gram positive bacteria (+ve), grow best at 30 °C with a pH range of 6.3-5.4, small colony size and creamy grey in colour on MRS agar, the selective media for lactic acid bacteria (Oxoid, 2004).

Biochemical test

The mutants isolates was found to react positively with Glucose, Galactose, Fructose, Lactose, Mannitol and Inulin as demonstrated in the method of Harrigan and McCance (1993), Sambrook et al. (1989), and Rserovska et al. (2002).

The reaction of Lb 30 and Lb 35 is also better on mannitol and Lb 35 is also better on inulin as compared to the wild type.

The protein content of mutants isolates Lb 20 shows a significant increase ($P<0.05$) from 7.37 ± 0.42 at zero day of fermentation to 11.48 ± 0.62 (5^{th} day of fermentation) and 11.52 (6^{th} day of fermentation). Similarly, there were significant increase ($P < 0.05$) in the crude protein content of mutants isolates Lb 25, Lb 30, and Lb 35 from 7.3 ± 0.42 at zero day to 11.21 ± 1.05, 5.80 ± 0.39 and 11.91 ± 0.82 (fourth day of fermentation), 9.72 ± 0.92, 9.29 ± 0.53, and 16.91 ± 0.73 ((fifth day of fermentation) and 12.18 ± 0.62, 9.72 ± 0.88 and 14.50 ± 0.52 (sixth day of fermentation) respectively.

The mutants isolates Lb 35 showed the highest crude protein content increase after the fifth day of fermentation of Irish potatoes (*Solanum tuberosum*).

Plasmid analysis

The result of Plasmid analysis showed the complete absence of any chromosomal element in the *Lactobacillus bulgaricus* strain.

Table 1: Identification of *Lactobacillus bulgaricus* Isolates from "kindirmo".

Characteristic	Isolate *L. bulgaricus*
Growth on MRS agar (pH 6.3)	+ve
Growth on MRS agar (pH 5.4)	+ve
Temperature of incubation	30 ˚C
Catalase activity	-ve
Gram reaction	+ve
Cell morphology	rods
Colony size	small
Colony shape	circular, irregular
Colony colour	creamy grey

Key : +ve = Positive ; -Ve = Negative

Table 2: The results for biochemical tests of *Lactobacillus bulgaricus* isolates from "*Kindirmo*".

Sugar	Reaction
Glucose	+++
Galactose	++
Frustose	+++
Lactose	+++
Mannitol	++
Inulin	++

Key: +++ strongly positive ++ = moderately positive + = positive.

Table 3: Characterisation of mutant isolates of *Lactobacillus bulgaricus.*

		Mutants	Isolates L.	bulgaricus
Characteristics	**Lb 20**	**Lb 25**	**Lb 30**	**Lb 35**
Growth on MRS agar (pH 6.3)	+ve	+ve	+ve	+ve
Growth on MRS agar (pH 5.4)	+ve	+ve	+ve	+ve
Temperature of Incubation	30 ^{o}C	30 ^{o}C	30 ^{o}C	30 ^{o}C
Catalase activity	-ve	-ve	-ve	-ve
Gram morphology	rods	rods	rods	rods
Colony size	small	small	small	small
Colony shape	Circular	Circular	Circular	Circular
Colony colour	creamy grey	creamy grey	creamy grey	creamy grey

Key: +ve = positive ; -ve = negative

Lb 20 = *Lactobacillus bulgaricus* exposed to UV-light for 20 seconds

Lb 25 = *Lactobacillus bulgaricus* exposed to UV-light for 25 seconds

Lb 30 = *Lactobacillus bulgaricus* exposed to UV-light for 30 seconds

Lb 35 = *Lactobacillus bulgaricus* exposed to UV-light for 35 seconds

Microscopic examination of the mutant isolates revealed all the above characteristics as demonstrated by Oxoid (2004).

Table 4: Biochemical characteristics of mutants of *L. bulgaricus.*

		Mutants	**Isolates**	***L.bulgaricus***
Sugar	**Lb 20**	**Lb 25**	**Lb 30**	**Lb 35**
Glucose	+++	+++	+++	+++
Galatose	++	+++	+++	+++
Fructose	+++	+++	+++	+++
Lactose	++	++	+++	+++
Mannitol	+++	+++	+++	+++
Inulin	++	++	++	+++

+++ = Strongly positive ; ++ = moderately positive ; + = positive
Lb 20 = *Lactobacillus bulgaricus* exposed to UV-light for 20 seconds ; Lb 25 = *Lactobacillus bulgaricus* exposed to UV-light for 25 seconds ; Lb 30 = *Lactobacillus bulgaricus* exposed to UV-light for 30 seconds
Lb 35 = *Lactobacillus bulgaricus* exposed to UV-light for 35 seconds

Table 5: Proximate analysis of unfermented and fermented content of Irish Potatoes with wild type *L. bulgaricus.*

	Periods in days				
Components of Proximate Analysis	**0**	**4**	**5**	**6**	**7**
Crude protein (%)	7.37±0.42	8.70±0.26	8.73±0.43	8. 03±0.53	7.64±0.33
Crude Fibre (%)	5.64±0.53	3.37±0.41	2.09±0.35	2.39±0.34	2.70±0.51
NFE (%)	16.15 ±1.21	14.63±0.45	3.88±0.50	5.72±0.33	5.73±0.41
Ash content (%)	5.83±0.41	11.15±0.38	10.25±0.40	9.83±0.32	10.88±0.41
Crude fat (%)	0.01±0.001	0.05±0.004	0.05±0.004	0.03±0.005	0.05±0.005
Moisture contents (%)	65.00±1.58	72.00±1.43	75.00±1.25	74.00±0.50	73.00±0.50

Note: All readings were means of three determinations (±SD). There was a significant decrease (P>0.05) in the carbohydrate 0-S and crude fibre contents from 0 day to the 5th day of fermentation and also a significant increase (P<0.05) crude fat and total ash content from zero days to seventh day of fermentation respectively. There was an increase but not significant (P>0.05) of crude protein.

Table 6: Protein analysis of fermented Irish Potatoes with mutants isolates.

Mutants isolates	**Fermentation periods in day**			
at periods in seconds	**0**	**4**	**5**	**6**
Lb 20	7.37±0.42	5.87±0.78	11.48±0.51	11.52±0.62
Lb 25	7.37±0.42	11.21±1.05	9.72±0.92	12.18±0.62
Lb 30	7.30±0.42	5. 80±0.39	9.29±0.53	9.72±0.88
Lb 35	7.37±0.42	11.91±0.82	16.91±0.73	14.50±0.52

Note: All readings were means of three determinations (±SD). Lb 20 = *Lactobacillus bulgaricus* exposed to UV-light for 20 secs; Lb 25 = *Lactobacillus bulgaricus* exposed to UV-light for 25 secs; Lb 30 = *Lactobacillus bulgaricus* exposed to UV-light for 30 secs. Lb 35 = *Lactobacillus bulgaricus* exposed to UV-light for 35 secs.

DISCUSSION

One of the major objectives of this research was to develop a simple biotechnological method that can be adopted and applied to improve the nutritional quality of some staple starchy foods such as Irish potatoes (*Solanum tuberosum*) with protein (amino acids) as there is high demand for essential amino acids for use in foods, feeds and in the pharmaceutical industries is

expanding (microbiology procedure.com). Research has also shown that it is genetically possible to "*tailor*" the microoganisms for the production of any microbial metabolite-vitamins, amino acids, or enzymes.

The isolation, identification and characterisation of the lactic acid bacteria isolate showed that the bacteria species was *Lactobacillus bulgaricus* (Tables 1 and 2) . The isolates of the wild type was found to react positively with glucose, Galactose, Fructose, Lactose, Mannitol and Inulin as demonstrated in the method of Harrigan and McCance (1993), Sambrook et al. (1989), and Rserovska et al. (2002) (Table 2). The mutant isolates also showed the same morphological and biochemical reaction similar to the wild type strains (Tables 3 and 4). The crude protein content of the fermented Irish potatoes with the wild type strain showed no considerable increase in the crude protein content from zero day to the seventh day of fermentation with a probable peak period of fermentation at the fifth day (5^{th} day) 8.73+0.43 g/100 g, (Table 5), whereas the mutant isolates generally showed a significant increase ($P<0.05$) in their crude protein content from zero day to the 6^{th} day. Mutants strains Lb 20, Lb 25, and Lb 30 shows a peak crude protein content at the 6^{th} day of fermentation (11.52+0.62, 12.18+0.62, and 9.72+0.88) and mutant strain Lb 35 shows its peak crude protein content at the 5^{th} day (16.91+0.73) respectively. The peak period of fermentation for the mutant isolates could not be determined by these results as the fermentation has not exceeded the 6^{th} day as it was in the case with wild type strain (Table 6).

The increase in crude protein content observed in the mutants isolate might probably be due to increase in conversion of carbohydrate to protein. This could be true, as the activities of the enzymes involved in transamination reactions (amino acids synthesis) use some of the glycolytic and TCA cycle intermediates such as phosphoglycerate, pyruvate, phosphoenolpyruvate and alpha ketglutarate as substrates in amino acids synthesis pathway. This agrees with the findings of Geo and Robert (2004), that raw material for amino acids synthesis can thus be obtained from glucose and the amino acids are synthesized at the cost of both energy (ATP) and reducing power (NADPH). One of such route is the enhanced activity of the enzyme phosphoenolpyruvate carboxylaze that converts phosphoenolpyruvate to oxaloacetate limiting the entrance of glycolytic intermediate into the tricarboxylic acid cycle (Lauaileche et al., 1996). This ascertion seems to concur with preliminary results of the amino acids content analysis of the unfermented and fermented Irish Potatoes with both wild type and mutant isolates (result not presented) indicating an increase but not significant ($P >0.05$) in the amino acids content of aspartic acids (synthesized from oxaloacetate) and glutamic acid (synthesized from alfa ketoglutarate).

The absence of plasmid DNA in the *Lactobacillus bulgaricus* strain appeared to rule out possible involvement of extrachromosomal gene activity in protein (amino acids) synthesis. This could be tenable as Plasmids or extrachromosomal elements are known to contribute in the synthesis of some macromolecules, e.g. proteins (amino acids).

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