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Production of Protoplasts from Yeast Cells (*Saccharomyces cerevisae*) Isolated from Palm Wine (*Elaeuis guinensis*) Using Snail Gut Enzyme from African Giant Snail (*Achatina achatina*)

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Authors' contributions

This work was carried out in collaboration between both authors. Author CCCO designed the study, wrote the protocol and the first draft of the manuscript. Author PNIN managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

Aims: The aim of this study was to investigate the use of locally sourced snail gut enzyme from African giant snail (*Achatina achatina)* for yeast protoplasts production.
Place and Duration of Study: Fresh undiluted palm wine was commercially acquired from Umuoke, Obowo in Imo State while African giant snails (*A. achatina) were bought from* Umuahia main market, Abia State, Nigeria. The study was carried out from June to september, 2013.
Methodology: The snails were starved for 7 days so as to concentrate their gut juice, carefully aspirated using sterile needles and syringes after unshelling the snails. All the extracts from twelve snails were pooled together and centrifuged at 1,500rpm for 20 minutes to remove larger particulates; the supernatant was collected dissolved in 0.1M acetate buffer (pH 5.5). Yeast cells (*Saccharomyces cerevisae) were grown in potato dextrose broth* and harvested at late exponential

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phase at 37ºC. 1g of the cell slurry were suspended into 10 ml of 0.1M phosphate buffer (7.2) containing 0.1% Tween 80(osmotic stabilizer). Crude snail gut enzyme extract in 0.1M acetate buffer (pH 5.5) was added to yeast suspension and incubated at 30°C for 120 mins with gentle shaking. The resulting protoplasts were centrifuged at (1000rpm) and suspended in 0.7Mol. NaCl solution as supplement osmotic medium. The effect of lytic incubation time, concentration and osmotic stabilizers were studied. The viability of the cells were assessed by regeneration method.

Results: A wet mount of the harvested yeast cells after staining with lactophenol cotton blue indicate presence of cell walls. However, after incubation of mixture of these cells with snail gut juice at 30°C for 120 mins. The effect of lytic incubation time for the protoplast production shown on Fig. 1, revealed optimal protoplast formation of 2.7 x 10^7/mL after 80mins of incubation. The enzyme concentration effect on protoplast production shown in Fig. 2 revealed that 100%v/v of gut juice from *A. achatina* was able to produce 2.0 x 10^7/mL yeast protoplast. The best supporting osmotic stabilizer during cell wall regeneration was observed with 0.5M sucrose.

Conclusion: This experiment therefore suggests a rapid, inexpensive and efficient procedure for yeast protoplasts production.

Keywords: Achatina achatina; protoplasts; Saccharomyces cerevisae.

1. INTRODUCTION

Protoplasts are widely used in biotechnological processes because they fuse readily [1]. Protoplasts of different microbial cells or species can be fused even if they are not closely linked taxonomically. With the help of protoplasts fusion, genetic information is transferred and recombinant features constructed [2]. Industrially, important cells such as yeasts have cell walls which make it difficult to fuse with cells of different species hence retarding the aim of improving some industrial products by producing good hybrids through genetic manipulation [3]. This problem is overcome by obtaining protoplasts and using them instead of using intact cells with cell walls. Palm wine is an alcoholic beverage produced by the natural fermentation of the sap of *Elaeis guineensis and Raphia hookeri* normally consumed in the tropics [4]. The unfermented fresh palm wine is a clean, sweet, colourless syrup containing about 10 - 12% about sugar mainly sucrose [5]. The sugar level rapidly drops due to fermentation process by microbial flora of the liquid and produces alcohol and other products [6]. It is widely consumed in Africa, Asia and Southern America particularly Southern Nigeria and some other parts of world including South India, Mayanmar and Mexico [7,8]. Palm wine is used for various purposes ranging from nutritional, medical, religious and social functions [9-11].

Previous study on the microbiology of *E. guineensis* has shown that several bacteria and yeast flora are involved in the fermentation process [5]. The nutritional content of palm wine is as a result of its probiotic content [12-14]. Palm wine contains good quantity of yeast although there are other organisms associated with it; [8,15] also reported the genetically and physiologically different isolated yeasts from palm wine. These variants include nine species of *S. cerevisiae*, three *Candida* species, one *Schizosaccharomyces* species and one *Kluyveromyces* species. The rapid development in area of biotechnology has shown that it is important to make use of these naturally occurring microbial flora to both improve the economy and assist the unemployed youth especially in the third world countries like Nigeria.

The aim of this work is to use available locally sourced materials (Yeast cells from alcohol beverage *E. guineensis* and enzyme from (*A. achatina*) to produce yeast protoplast that can be useful to industries and subsequently create wealth to most unemployed graduates in the field of biotechnology.

2. MATERIALS AND METHODS

2.1 Sample and Pure Cultures Collection

Fresh and undiluted palm wine and African giant snails were acquired commercially from Umuoke, Obowo in Imo State, Nigeria and Umuahia main market in Abia State, Nigeria respectively.

2.2 Microbial Analysis

The method used was as described by [4] with slight modification 1mL aliquot of fresh and undiluted palm wine commercially sourced from

Umuoke, Obowo in Imo State, Nigeria, was serially diluted. 1mL was pour-plated in triplicate using Potato Dextrose Agar (PDA) containing 0.1% (w/v) lactic acid. The plates were incubated at 28°C±2°C for 48 hrs. Colonies were sub cultured unto fresh PDA using the streak method. Each colony was confirmed to be yeast by examining it under a light microscope using oil immersion objective after staining with lactophenol-in-cotton-blue dye. The isolated yeasts were characterized based on physiological, morphological and biochemical characterization as described by [16,17]. Pure isolates were placed on PDA slants and stored at 4ºC until needed.

2.3 Enzyme Extraction

The snails were starved for 7 days so as to concentrate their gut juice which is brownish in colour and carefully aspirated using sterile needles and syringes after unshelling the snails. An average sized snail weighing 130g contains about 3mls of gut juice. All the extracts from twelve snails were pooled together and centrifuged at 1,500rpm for 20 minutes to remove larger particulate components; the supernatant was collected by decanting and dissolved in 0.1M acetate buffer (pH 5.5) according to [18].

2.4 Protoplast Production

The method used in production of yeast protoplast was as described by [15] with slight modification. Pure culture of *S. cerevisae was* inoculated in 50 ml of *potato dextrose broth* in a 250 ml conical flask with a final concentration of 1x 10^7 cells/ ml and incubated with shaking at 200 rpm until late exponential phase (24 hrs) at 37°C. After the incubation the yeast cells were harvested by filtration process through sterile muslin. 1g of the cell slurry was re-suspended into 10 mL of 0.1M phosphate buffer (7.2) containing 0.1% Tween 80(osmotic stabilizer). Crude snail gut enzyme extract at various dilutions (20, 40, 60, 80, 100 %v/v) in 0.1M acetate buffer (pH 5.5) was added to yeast suspension and incubated at 30°C for 120 mins with gentle shaking using shaker incubator at 100rpm. Protoplasts formation was examined by observing for evidence of protoplast using small sample during lytic digestion at different time after 30, 60, 90 and 120 min. The presence of residual cell wall was monitored by reacting the suspension with lactophenol cotton blue. Finally, the filtrate was centrifuged at 1000 rpm for 15mins and protoplasts were collected, washed in 0.7M NaCl solution twice and re-suspended in 0.7M NaCl solution. Finally, the protoplast was observed by microscope and counted using hemacytometer [19]. The viability of the cells were assessed by regeneration method according to [20,21] by plating 1ml of serial 10 fold dilution of protoplast suspension in different osmotic stabilizers (sucrose, KCl, and NaCl at 0.5M or 0.7M)in potato dextrose agar. A control was done in PDA medium, with no osmotic stabilizer, to calculate the regeneration frequency. Plates were incubated at 28°C until colonies formation on the surface of the medium.

3. RESULTS AND DISCUSSION

A wet mount of the harvested yeast cells after staining with lactophenol cotton blue indicate the presence of cell walls. After incubation of mixture of cells with snail gut juice at 30°C for 120 mins, the cell walls were totally digested exposing the yeast protoplasts. The effect of lytic incubation time for the protoplast formation was demonstrated in Fig. 1. It was shown that optimum protoplast formation (2.7 x 10^7/mL) was observed after 180mins of incubation using crude enzyme (gut juice). Nwachukwu et al. [22] reported the production of yeast protoplast of palm wine origin in production of high yield ethanol. There was higher quantity of protoplast release compared to studies of Ezeronye and okerentugba [15] who reported 1.54 x 10^6/ mL of protoplast from cells exposed to crude enzyme and incubated for 180mins. The variation in enzyme activity may be associated with some environmental factors such temperature, pH and water retention capacity during the breeding of the snail.

Similarly, the effect of enzyme concentration on protoplast formation shown in Fig. 2 revealed that 100%v/v of gut juice from *A. achatina* was able to produce 2.0 x 10^7/mL of yeast protoplast. This compared favorably withthe result of Ezeronye and Okerentugba [15] who reported the release of 1.6 x 10^6/mL protoplast of *S. cerevisiae* with 100% enzyme concentration.

The best supporting Osmotic stabilizer for *S. cerevisiae* was demonstrated at 0.5M sucrose with 87% protoplast regeneration frequency Fig 3. Osmotic stabilizers varied with the fungi, 1.0M sorbitol and 0.8M mannitol was reported to have best supported regeneration *of S. cerevisiae* and *Candidum tropicalis* [15], 0.5M sucrose was also

reported as a good stabilizer for *Crinipellis perniciosa* [23]. Similarly 0.5M sucrose exhibited the best stabilizer in *Epulorhizarepens* with 8.5% regeneration frequency [24].

Sucrose utilization in yeast cell mostly involves extracellular enzyme invertase (β-D-fructosidase) but there are reports of *S. cerevisiae* strains that have two forms of invertase (both external large invertase, and internal small invertase) [25]. However, some strains of *S. cerevisiae* have been reported to transport sucrose directly into the cell [26,27]. From this study the use of yeast protoplast makes it easier for sucrose to be a better stabilizer during regeneration process.

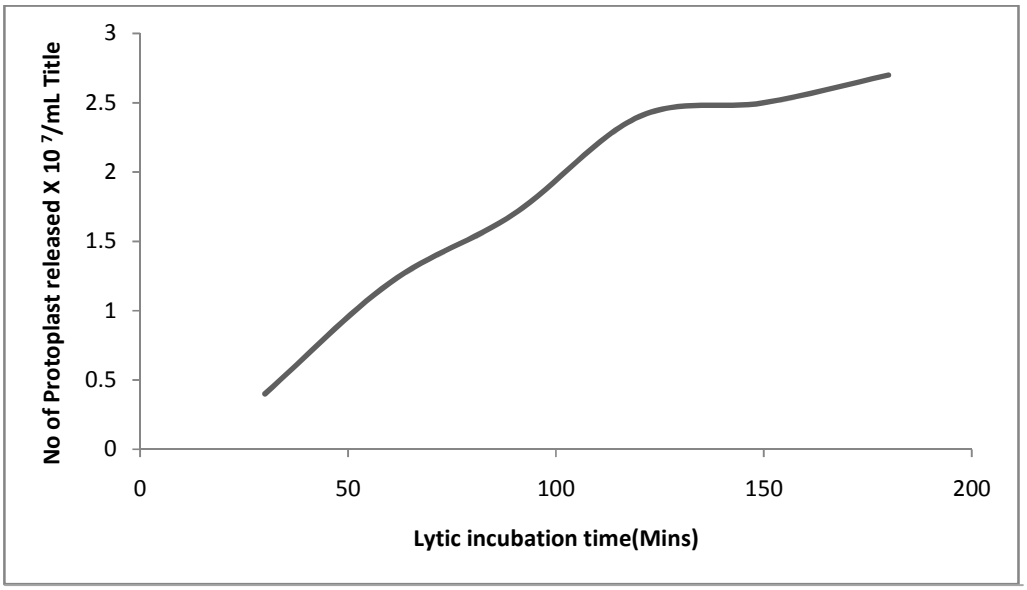

Fig. 1. Effect of lytic incubation time on production of yeast protoplast

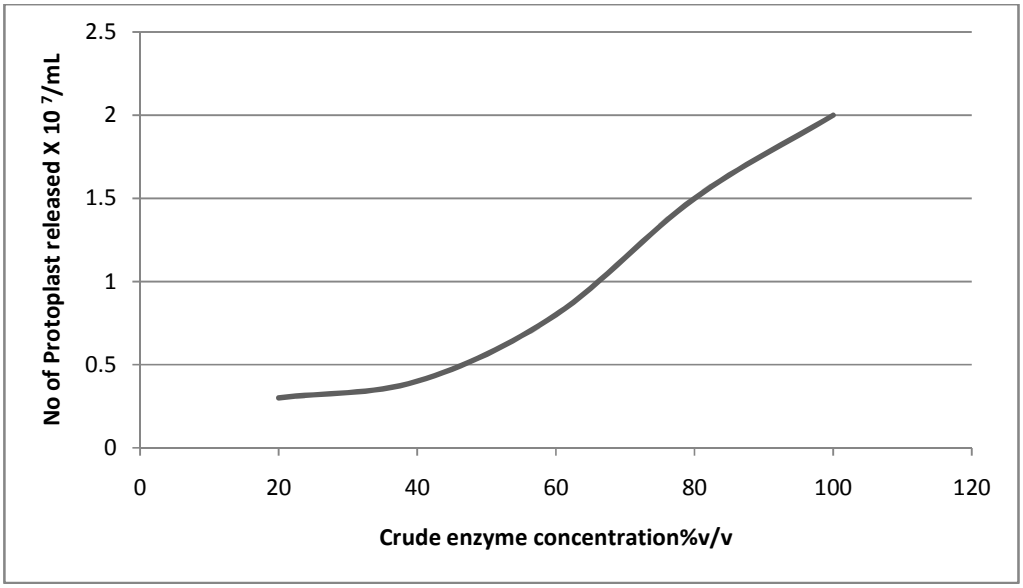

Fig. 2. Effect of concentration of crude enzyme (Gut Juice) on yeast protoplast production

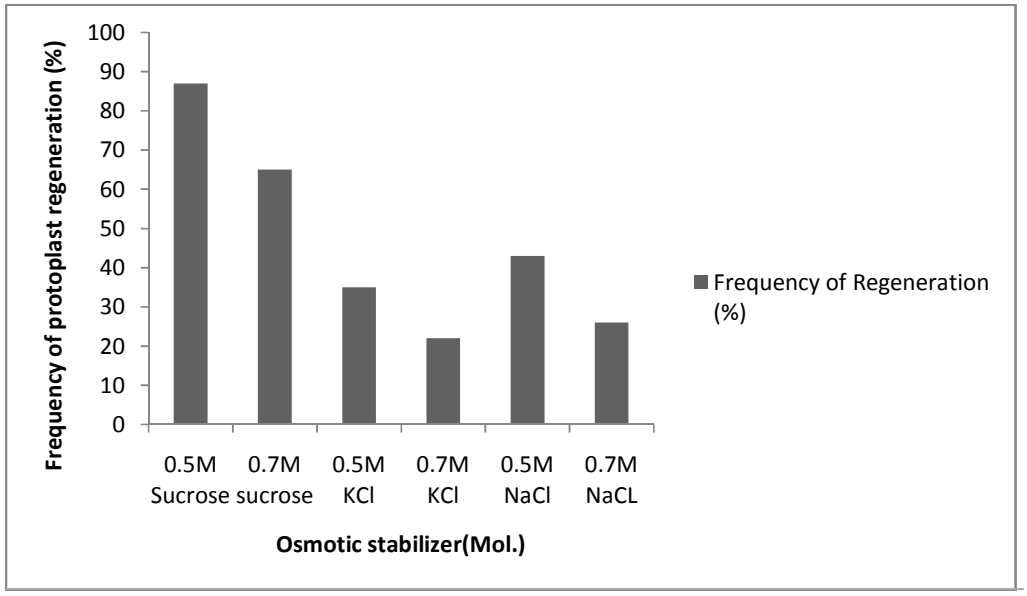

Fig. 3. Effect of osmotic stabilizers on protoplast regeneration

4. CONCLUSION

There is presence of enzymes that digest cell walls of yeast cells in the gut of African giant snails leaving them without walls (protoplasts). More than 80% of the protoplast regenerated a fresh cell wall after 10-15days at 28°C.

This experiment therefore suggests a rapid, inexpensive and efficient procedure for yeast protoplasts' production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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