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Isolation and Identification of Indigenous Yeast Strain and Its Potential for Yeast Extract Production

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Abstract—The growing demand for protein-rich food forces humans to search for alternative protein sources. Yeasts are among the preferred candidates due to their well-balanced source of amino acids and vitamins. The present study aimed to identify an indigenous yeast strain that can be used as a protein source. Yeast strains were isolated from Palmyrah toddy sediments collected from different regions in the Jaffna Peninsula. One yeast strain, named A3, was selected as a potential strain based on the maximum cell size and optical density among the 55 yeast strains isolated. The biochemical analysis and sequencing of the ITS region (including 5.8S rRNA gene) and the LSU rRNA gene D1/D2 domains confirmed the yeast isolate as a strain of *Saccharomyces cerevisiae*. The optimum growth conditions for the yeast strain were determined using Taguchi L_{16} orthogonal array. Yeast cell autolysis was conducted with and without papain, and the yield of soluble matter and total protein were measured. The strain yielded a maximum of 70.7 % soluble mater and 56.9 % of total protein when autolysis with papain. The yield is significantly higher ($p<0.05$) than obtained with the baker's yeast used in the control experiment.
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Keywords— Palmyrah toddy, *Saccharomyces cerevisiae*, single-cell protein, yeast.

I. INTRODUCTION

The increasing population in developing countries is expected to create a high demand for protein-rich food by 2050 (Boland *et al*., 2013). Therefore, new initiatives will be required to produce the necessary quantities of high-quality protein. Various microbes and algae can be good sources for protein, preferably those containing more than 30% protein in their biomass and providing a healthy balance of essential amino acids (Ritala *et al*., 2017).

Yeasts are significantly valuable in human culture, and they are widely used to produce alcoholic beverages and bakery products. Since the yeast contains 30–60 % protein, they have been used as single-cell protein (Anupama and Ravindra, 2000; Nasseri *et al*., 2011). Spent brewer's yeast (*Saccharomyces cerevisiae*) have been sold for more than a century in yeast extracts such as Marmite® (Unilever and Sanitarium Health Food), Vegemite® (Bega Cheese Ltd.), Cenovis® (Gustav Gerig AG), and Vitam-R® (VITAM Hefe-Produkt GmbH). The yeast extracts are also a good source of thiamine, riboflavin, biotin, niacin, pantothenic acid, pyridoxine, choline, streptogenin, glutathione, folic acid and p-aminobenzoic acid (Frazier and Westhoff, 1988). The extract obtained from yeast cultures is widely used in the food industry to enhance or provide a meaty flavour to food products (Tanguler and Erten, 2008).

Mostly, the yeast extract is prepared from baker's yeast or spent brewer's yeast by autolysis. At an elevated temperature, endogenous enzymes degrade yeast cells. The yield of extract in standard autolysis usually varies between 19 to 56% for different types of yeast (Sugimoto, 1974; Kollar *et al*., 1991; Vukasinovic Milic *et al*., 2007). Extract yields can sometimes be improved by adding commercial food-grade proteases such as papain (Verduyn *et al*., 1999). Several studies have been conducted to optimise factors that influence protein extraction from different types of yeasts (Hongpattarakere and H-kittikun, 1995; Taran and Bakhtiyari, 2013; Lapeña *et al*., 2020).

Palmyrah toddy is a sweetish, milky white, vigorously effervescent, mildly alcoholic beverage obtained from Palmyrah palm (*Borassus flabellifer* L.). Since ancient times, toddy has been consumed by the indigenous people, similar to wine in Europe. The toddy is collected by tapping the male or female inflorescence of the Palmyrah palm. The sweet sap oozed from inflorescence is collected in pods, and the sap undergoes spontaneous fermentation by air-borne yeast isolates. The yeast sediment at the bottom of the fermentation tank has been considered as an inconvenient waste, and it is left out as waste material. About 30 years ago, a study conducted based on yeast's biochemical and morphological characteristics revealed the association of *Saccharomyces cerevisiae*, *Kloeckera apiculata*, *Schizosaccharomyces pombe* and *Saccharomyces chevalieri* in the sediments of the toddy (Theivendirarajah and Chrystopher, 1987). The present study aimed to isolate and identify a potential yeast isolate from the toddy sediment and to increase protein yield from that isolate by optimising the growth and autolysis on a laboratory scale.

II. MATERIALS AND METHODOS

A. Toddy Sample Collection and of yeast isolation

Toddy samples with yeast sediments were collected from Palm development cooperative societies (PDCS) in nine different regions, namely Maruthankeny, Chunnakam,

Achchuvely, Chavachcheri, Manipay, Pandaththarippu, Nelliyady, Kodikamam and Kayts in Jaffna district, Sri Lanka. In each PDCS, five different samples were collected between January to March 2018. The yeast sediments were serially diluted up to a notional 10^{-4} dilution in sterile saline (0.85% NaCl). The suspension was transferred on yeast extract peptone dextrose agar (YEPDA) medium (1 L medium contain yeast extract 5.0 g, peptone 10.0 g, dextrose 20.0 g and agar 2.5 g) by spread plate method, and the plates were incubated at 28 ºC for 3-5 days. Morphologically distinct fifty-five colonies were sub-cultured on a fresh medium of the same composition.

B. Selection of potential yeast isolate based on cell size and optical density in suspension

The isolates were grown in yeast extract peptone dextrose (YEPD) broth (1 L medium contains yeast extract 5.0 g, peptone 10.0 g, dextrose 20.0 g) at 28 ºC in a shaker at 100 rpm. After 48 h of incubation, each isolate's cell diameter or length was measured using an ocular micrometre. In addition, the optical density (600 nm) of the aliquots of yeast cell suspensions was measured using UV – Vis spectrophotometer (Thermo Scientific™ GENESYS™ 10S) after three days of incubation. Finally, an isolate with the largest cell size and highest density in suspension was selected for further screening and identification.

C. Biochemical characterisation of the yeast isolate

The isolate was tested for carbohydrate fermentation, carbon assimilation, temperature tolerance and urease production. All the experimental procedures were followed as described by Kurtzman et al. (2011a). For the carbohydrate fermentation, the isolate was inoculated in YEP broth with bromothymol blue and different sugars, namely sucrose, D-galactose, glucose, lactose, maltose and raffinose. Carbon assimilation of the yeast isolate was tested by inoculating in yeast nitrogen base medium, which was incorporated with different carbon compounds (Glucose, mannitol, ribose, galactose, myo-inositol, lactose, xylose, inulin, sucrose, D-arabinose, maltose, ethanol, methanol, glycerol and L-arabinose). The isolate was grown on YEPD medium at temperatures 19, 25, 30, 37 and 45°C to find optimum growth temperature. Similarly, Christensen urea agar medium (with 20 %) at pH 6.8 was used to test the hydrolysis of urea.

D. Molecular identification of the yeast isolate

D.1. DNA extraction

DNA isolation was performed as described by Senses-ergul *et al*. (2006). The yeast isolates cultured on tryptone glucose yeast extract (TGY) agar for 24 h at 30 °C was suspended and washed in 1 mL of sterile distilled water. Then the cells were collected by centrifugation at 14,000 rpm for 2 min and 200 µL of breaking buffer (2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA). Glass beads (diameter 0.45–0.50 mm), 200 µL of buffered phenol, chloroform and isoamyl alcohol (25:24:1, v/v) were added. After vortexing for 3 min, 200 µL TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) was added to the mixture. It was followed by vortexing for 1 min and centrifugation at 14,000 rpm for 5 min. The upper phase was pipetted into 1 mL of ice-cold 96% ethanol and mixed. The precipitated nucleic acids were collected by centrifugation at 14,000 rpm for 5 min, and the pellet was redissolved in 50 µL TE buffer. For the digestion of RNA, 3 µL RNAse (10 µg/mL) (Sigma) was added, and the mixture was incubated at 37 °C for 30 min. After incubation, the DNA was precipitated in 1 mL of ice-cold 96% ethanol and centrifuged at 14,000 rpm for 5 min. The pellet was redissolved in 50 µL TE buffer and stored until use at -20 °C.

D.2. PCR amplification and sequencing

The sequences of the ITS region (including 5.8S rRNA gene) and the LSU rRNA gene D1/D2 domains were PCR amplified from the genomic DNA of potential yeast strain using primers NL1 and NL4 (Kurtzman and Robnett, 1998), and ITS1 and ITS4 (White *et al*., 1990), respectively. The PCR products were sequenced using forward and reverse primers by an automated Sanger sequencing service (Macrogen, Korea). Identity searches for the sequences were carried out using the BLAST (Altschul *et al*., 1990) search available in the NCBI. Sequences generated as part of this study are deposited in GenBank. Additional sequences were obtained from Kurtzman and Robnett (2003). All the sequences were aligned using the MUSCLE (Edgar, 2004) algorithm. Phylogenetic trees were reconstructed from the sequences using the Maximum Likelihood algorithm in MEGA software version 7.0 (Kumar *et al*., 2016). Confidence levels of the clades were estimated from bootstrap analysis based on 1000 replications.

E. Optimisation of growth conditions of the potential yeast strain

Taguchi methodology was used to find the optimum growth conditions. An L_{16} orthogonal array (OA) in four levels was used, which consisted of 16 different experimental trials (Elizalde-González and García-Díaz, 2010). Five selected factors, namely the amount of glucose, yeast extract and peptone, level of pH and inoculum size, were selected for medium optimisation. Level 1 for each factor was fixed at a low value, considering the factors' role in yeast growth in the fermentation medium. In contrast, levels 2 and 3 were deemed intermediate levels for yeast growth. Level 4 of each factor was selected at a relatively higher value range. All the trials were conducted at 30 °C for two days at 150 rpm, finally, OD was measured.

All five selected factors, their assigned levels and the experimental design are listed in Table 1. The design for the L_{16} OA was developed and analysed using "MINITAB 19" software.

Table. 1: Selected factors and their assigned levels for the optimization of fermentation media

Factors	Level 1	Level 2	Level 3	Level 4
pH	2.5	4.5	5.5	6.5
Inoculum size (µl/100ml)	250	500	750	1000
Yeast extract (g/100ml)	0.25	0.5	0.75	1.0
Peptone (g/100ml)	0.5	1.0	1.5	2.0
Glucose (g/100ml)	1.0	2.0	3.0	4.0

F. Preparation of yeast extract from identified potential yeast isolate

The potential yeast strain was cultured in 250 ml Erlenmeyer flasks containing 100 ml medium under optimum growth conditions which were determined in a growth optimisation experiment. The inoculated flasks were incubated in an orbital shaker at 30 °C for two days at 150 rpm. The yeast cells were harvested by centrifugation at 8000 rpm for 10 min at ambient temperature. The pellet was re-suspended in phosphate-buffered saline and centrifuged again at the same conditions. The resulting pellet volume was estimated, and aliquots of 15% (w/v) suspension of the yeast slurry in sterile deionised water was prepared under aseptic conditions in a 250 mL flask. The yeast cell disruption was carried out in two different methods. In one method, the aliquots were subjected to autolysis, and the pH level was adjusted to 6.0 with 1N NaOH. For the second method, papain [1 % (w/v) final concentration] (Papain from papaya latex, Sigma-Aldrich) was added to the aliquots, and the pH was adjusted to 6.0. The flasks were placed in a shaking water bath at a constant temperature of 55 °C at 100 rpm. After 24 h, the flasks were heated at 85 °C for 15 min then cooled down to room temperature and finally, centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was used for chemical analysis. In a control experiment, baker's yeast was used instead of the yeast strain identified in this study. The total solids in the fresh yeast and yeast extract were determined by the dry weights following drying at 105°C until a constant mass was achieved. The total solids content was expressed as a percentage of solids recuperated in the yeast extract with respect to the total solids present in the fresh yeast. The total crude protein content was determined by the Lowry method (Lowry *et al*., 1951).

III. RESULTS AND DISCUSSION

In this study, fifty-five different yeast strains were initially isolated from yeast sediments based on the diversity in the colony and cell morphology. The mean cell size of the isolated strains ranged from 4.0 µm to 7.8 µm after 48 h of incubation. The yeast cell suspensions' mean optical density (OD600nm) was between 0.1 and 3.99 in three days of incubation (Figure 1). Since the cell size is a reasonable estimation of the protein content of yeast (Alberghina et al., 1998), the strain that had the largest cell and maximum optical density was selected as potential yeast for further study. The strain A3, isolated from the toddy sample collected in Achchuvely, showed the highest diameter (7.5 ± 0.3 µm) and density (3.49 ± 0.5) at the tested growth conditions. The strain showed fermentation in a medium incorporated with sucrose, D-galactose, glucose, lactose or maltose. But it failed to grow in Raffinose medium (Table 2). Similarly, carbon sources such as glucose, galactose, inulin, sucrose, maltose, ethanol and L-arabinose were assimilated. However, growth was not observed in the assimilation medium incorporated with mannitol, ribose, myo-inositol, lactose, xylose, D-arabinose, glycerol. The strain did not show growth and utilisation of urea. Among the different tested growth temperatures, the strain showed maximum growth at 30 °C.

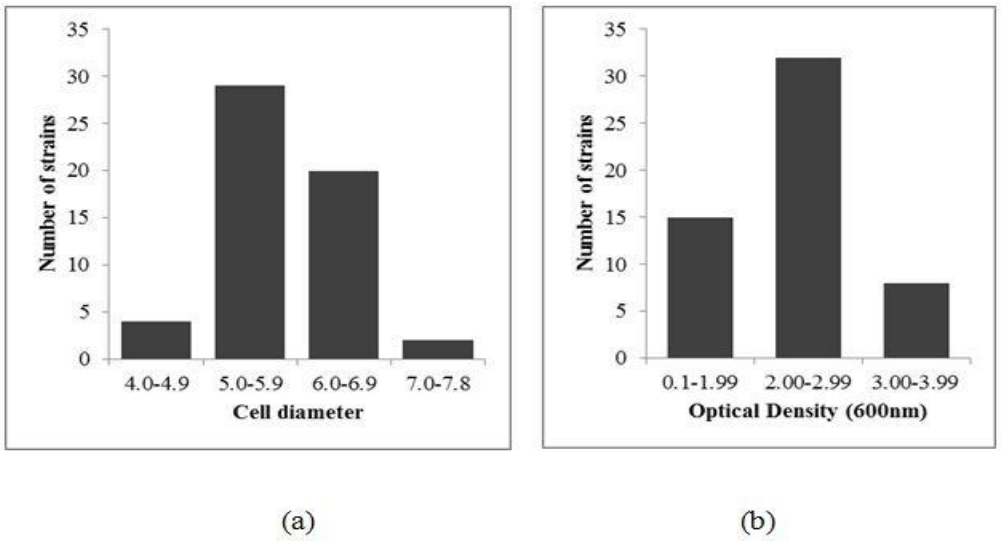

Fig. 1: Characters used to screen isolated fift five yeast strains. (a) Cell diameter (µm) and (b) optical density.

Even though biochemical characteristics have been used in yeast taxonomy, identification of yeast species entirely based on morphological traits and physiological abilities is laborious and time-consuming (Carvalho *et al.*, 2005; Kawahata *et al.*, 2007). Furthermore, it is also difficult to distinguish some closely related species. In contrast, gene sequence comparisons offer the opportunity to resolve closely related species, as well as more distantly related taxa. As the ITS sequences are commonly used to determine species, often in conjunction with those from the D1/D2 LSU rRNA gene (Kurtzman *et al*., 2011b), both of these regions were amplified in the present study and sequenced. Amplification of the ITS region (including 5.8S rRNA gene) and the LSU rRNA gene D1/D2 domains of the potential yeast strain A3 yielded 841 bp and 615 bp PCR products, respectively. The sequences were deposited in the GenBank database with accession numbers MT322849 (for ITS region) and MT322857 (for D1/D2 domains). DNA homology search in BLAST showed >99% sequence identity (with 100% query coverage) with *Saccharomyces cerevisiae* (baker's yeast).

Phylogenetic analysis of the two sequences with sequences retrieved from the GenBank database demonstrated that the strains were clustered closely with *Saccharomyces cerevisiae* strains. Both clades were obtained with high (> 80%) bootstrap support values (Figure 2 and 3). Based on the above results, the potential yeast strain A3 was confirmed as a strain of *Saccharomyces cerevisiae*.

Table. 2: Characteristic feature of the selected yeast strain

Character assessed	Observation in the isolate
Colony characters	
Colour	White
Form	Circular
Margin	Entire
Surface	Smooth
Texture	Mucoid
Elevation	Convex
Cell morphology	
Shape	Oval
Size	7.5 ± 0.3 µm
Carbohydrate fermentation	
Sucrose, D-galactose, Glucose, Lactose and Maltose	Positive
Raffinose	Negative
Carbon assimilation	
Glucose, Galactose, Inulin, Sucrose, Maltose, Ethanol, L-arabinose	Positive
Mannitol, Ribose, Myo-Inositol, Lactose, Xylose, D-arabinose, Glycerol	Negative
Urea hydrolysis	Negative
Growth temperature	30 °C
OD_{600nm}	3.49 ± 0.5

It is well documented that the macromolecular composition of growing microbial cells varies in relation to the growth rate (Villadsen and Nielsen, 2011; Zakhartsev and Reuss, 2018). Therefore, Taguchi L16 orthogonal experiment design was used to determine the growth parameters needed for the optimal growth of strain A3. The model was highly significant considering its r^2 = 0.973. Based on the results obtained in this analysis, the effect of each test media component on the growth of the yeast can be ranked as pH > glucose > inoculum size > peptone > yeast extract (Table 3). The highest delta value (0.4125) was obtained for the pH of the medium when the pH value was increased to 5.5. The ANOVA results also revealed pH of the medium was the significant factor (p= 0.011) compared to other tested factors.

Figure 4 illustrates the optimum conditions required for yeast growth. The optimum composition for 100 mL medium included 3 g of glucose, 2 g of peptone, and 0.75g of yeast extract. The pH of the medium needed to be 5.5 and the initial inoculum was 500 µl. The confirmation experiment is a crucial step and is highly recommended by Taguchi to verify the experimental results (Ross, 1996).

In this study confirmation experiments were conducted by using optimised factors and levels. The average of the results from the confirmation experiment is compared with the predicted average based on the parameters and levels tested. The obtained OD600 was 7.89 in the confirmation experiment while the amount of predicted value of the design was 8.28. The quantity of resulting yeast extract depends on factors such as cell disruption method, temperature, pH, duration of disruption and amount of yeast cell.

Table. 3: Effect of growth factors and their assign level on the mean growth (OD_{600}) of yeast strain

Level	pH	Inoculum size	Yeast extract	Peptone	Glucose
1	0.2508	0.5172	0.5198	0.4543	0.4630
2	0.6315	0.6178	0.5695	0.5685	0.5045
3	0.6633	0.5713	0.5873	0.5755	0.6262
4	0.6355	0.4748	0.5045	0.5827	0.5872
Delta	0.4125	0.1430	0.0828	0.1285	0.1632
Rank	1	3	5	4	2

In this study, cell disruption was carried out by the autolysis method with or without papain, and baker's yeast was used as a control. The autolysis temperature, pH and papain concentration were not optimised in this study. But these

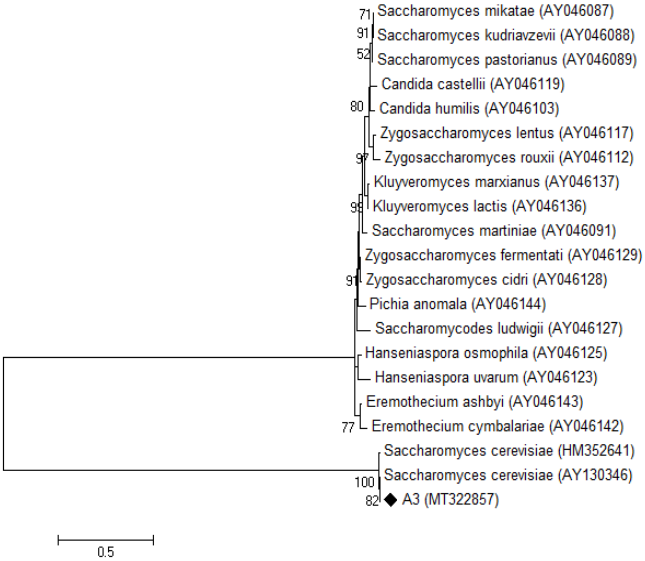

Fig. 2: Phylogenetic tree based on the LSU rRNA gene D1/D2 domains sequences showing the position of potential yeast strain A3 among related species. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. Bootstrap values >50% (based on 1000 replicates) are given at branch points. GenBank accession numbers are given in parentheses. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

parameters were selected from several previous reports (Conway *et al*., 2001; Vukasinovic Milic *et al*., 2007; Dolińska *et al*., 2012). Papain can be used in concentrations that go from 0.1% to 2.5% at temperatures in the 50–60°C range and slightly acidic pH ~6. Therefore in the present study, 1% was used for digestion. The result obtained from the experiment is given in Table 4. High solid recovery of 70.7 % was obtained by papain treatment of yeast strain A3. This solid recovery was higher than that of several previous reports (Verduyn *et al*., 1999; Chae *et al*., 2001; Vukasinovic Milic *et al*., 2007) with active baker's yeast or brewer's yeast and obtained using a combined treatment of proteolytic enzymes.

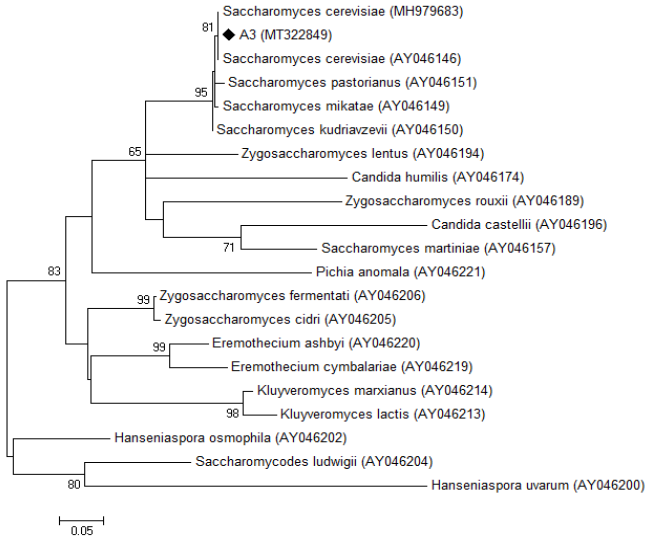

Fig. 3: Phylogenetic tree based on the ITS region (including 5.8S rRNA gene) sequences showing the position of potential yeast strain A3 among related species. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. Bootstrap values >50% (based on 1000 replicates) are given at branch points. GenBank accession numbers are given in parentheses. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

In the yeast extract preparation, the yeast strain used strongly affected the recovery of the cell constituent. The strain A3, isolated from toddy sediment, yielded more total-soluble matter and total protein than the control baker's yeast. The addition of 1% papain resulted in maximum yeast extract, which contained a maximum protein content of 56.9 mg/mL. This result is equal to a previous report with active baker's

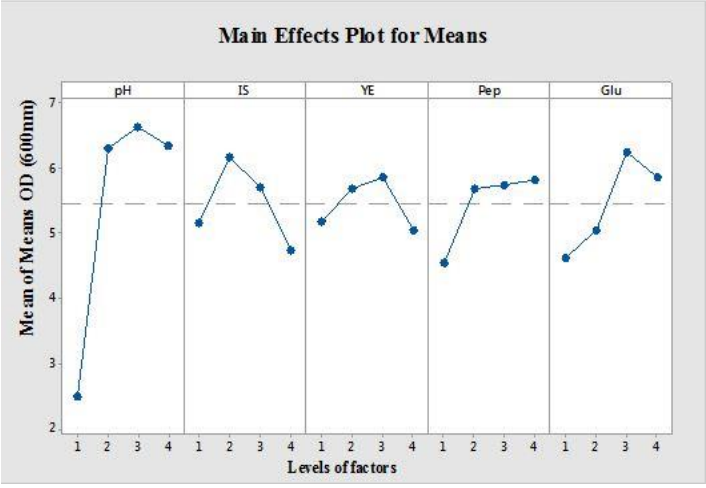

Fig. 4: The main effects of each factor on growth of the yeast strain. The detail of each level of factor is given in Table 1. pH- pH of the initial medium; IS – inoculum size; YE – yeast extract; Pep – peptone; Glu – glucose.

yeast treated with 2.5 % papain (Vukasinovic Milic *et al.*, 2007). Consequently, a process using an indigenous yeast strain and papain was developed to produce high-protein yeast extract. Further research is needed to develop an economically viable yeast production process based on industrial side streams or agricultural wastes as substrates.

Table. 4: Effect of yeast cells disruption

Method of cell disruptions	Soluble matter (%)		Protein (mg/mL)	
	Strain A	Baker's yeast	Strain A	Baker's yeast
Autolysis	67.6± 1.5	64.6± 2.1	54.2 ± 1.5	51.7 ± 1.6
Autolysis + papain	70.7± 1.2	68.7± 1.3	56.9 ± 1.9	53.9 ± 1.9

IV. CONCLUSION

The present study was conducted to identify yeast strain in toddy sediments with the potential to use in single-cell protein production or yeast extract preparation. The isolated strain A3, a strain of *Saccharomyces cerevisiae*, has been identified as a potential yeast strain. Under optimised conditions, the yeast strain A3 can yield more protein than baker's yeast. Furthermore, autolysis coupled with exogenous enzyme papain enhanced protein extraction.

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DISCLOSURE STATEMENT

The authors reported no potential conflict of interest

AUTHOR'S CONTRIBUTION

ECJ supervised the research project and conducted experimental procedures for yeast identification. SM carried out isolation, optimisation and autolysis experiments. ECJ and SM drafted the manuscript. SSV coordinated the research activities. All authors read and approved the final manuscript.

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