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Production of mini-food by *Aspergillus niger*, *Rhizopus oryzae* and *Saccharomyces cerevisiae* using orange peels

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Abstract

In this study, an evaluation of the possible use of orange peels (OP) as substrate to produce single-cell protein and mini-food by Aspergillus niger, Rhizopus oryzae and Saccharomyces cerevisiae was performed. The role of several fermentation parameters including moisture level, bed loading, initial spore count, incubation period, agitation rate, initial pH, incubation temperature, and carbon and nitrogen nutrition was investigated. Efficiency of 0.4 M NaOH, 0.3 M NaOH and Tris-HCl buffer (pH 9) for extraction of protein from the formed biomass of A. niger, R. oryzae and S. cerevisiae respectively, was demonstrated. Profiles of essential amino acids of the single-cell protein (SCP) compared favourably with FAO standards. RNA levels in the yielded protein were successfully reduced to 1.7%, 1.9%, and 1.4% (w/w).

Keywords: Single-cell protein – mini-food – *A. niger* – *R. oryzae* – *S. cerevisiae* – orange peels.

Introduction

The United Nations reported that over 1 billion people currently do not have access to adequate amounts of food due to several factors such as world overpopulation, decrease in agricultural production and depletion of fish stocks [9, 46]. Intensive production of food, and protein from animal sources, requires large areas of land and nitrogenous fertilizers which in turn can lead to a variety of environmental and health problems. Consequently, single-cell protein (SCP) technology arose as a promising alternative to the conventional methods of protein production [27]. SCP refers to dehydrated microbial cells of algae, bacteria, yeasts, molds and higher fungi, grown in mass culture, that are harvested for animal or human food due to their high protein content [1]. Since many of the SCP-producing organisms are multicellular and, as their biomass consists of more than just protein, it has been suggested that an alternative name such as novel protein or mini-food be used to better describe this product [1]. The use of fermentation processes to produce SCP have several advantages such as reduced land requirements; independence from seasonal variation, climatic conditions, or geographic location; ease in control of product quality and quantity; a wide variety of methodologies and raw materials; and use of microorganisms that are highly efficient and productive [8, 39]. Aside from procedural considerations, the fact that SCP products are very protein-rich, with a wide spectrum of amino acids and vitamins, but low concentrations of fat and are cholesterol-free, highlights their potential as suitable candidates for many versatile applications. Some such examples of these applications include both animal nutrition (e.g. in poultry, pigs and fish), and the improvement of the nutritive value of baked products, soups, ready-to-serve meals and diet products [25, 28]. SCP is gaining in popularity daily, not only in developing countries suffering from food shortages, but also among vegetarians, athletes, and patients in recovery, where it serves as a food supplement [9].

The safety concerns throughout production of SCP must be addressed by proper selection of microorganisms to be those classified as GRAS (generally regarded as safe), appropriate substrate selection and by performing the necessary toxicological studies. The

cost effectiveness of the product meanwhile, could easily be controlled by using low-cost substrates, which account for about 62% of the total product cost, and by selecting a suitable fermentation protocol [44]. In addition to the numerous advantages of solid-state fermentation (SSF) over submerged fermentation (SMF), which include its capacity to use waste as a substrate and converting this into added nutritional market value with high efficiency and lower downstream processing effort [3, 31, 48], it is a relatively simple and potentially adaptable technology that is applicable to use in rural situations in developing countries [33]. In recent years, a number of agricultural, agro-industrial and industrial wastes such as those originating from fruit-processing (orange, mango, mandarin and banana) and coffee-pressing; straw (barley, rice, corn and wheat); cotton stalks; bagasse (cassava and sugar cane), and some natural gases and petroleum hydrocarbons have all been used to produce SCP in various microorganisms [9, 27, 28, 33]. The use of such cheap and readily available substrates is desirable as it lowers the cost of production and reduces sanitary hazards resulting from waste and pollution problems while also conserving natural resources [41].

The most famous microorganisms in production of SCP are *Fusarium venenatum* (formerly *F. graminearum*) strain A3/5 and *Candida utilis* (formerly *Torulopsis utilis*), with their SCP is now being marketed under the names "Quorn™" and "Torutein" respectively [28]. Other filamentous fungi mostly belonging to the genera *Aspergillus, Chaetomium, Paecilomyces, Penicillium,* and *Trichoderma,* and yeasts belonging to the genera *Candida, Kluyveromyces* and *Saccharomyces,* in addition to a few bacterial species, have also been reported as SCP producers [1, 28]. Fungi and yeasts have several advantages over bacterial and algal cells through their ease of harvest, lower nucleic acid content, high lysine content and ability to grow at acidic pH levels. However, the most important advantage is familiarity and acceptability due to their long history of use in traditional fermentation, which dates back to the time of the Ancient Egyptians, through the employment of *Saccharomyces cerevisiae* in baking and ethanol fermentation [12, 27]. Moreover, growth of fungi and yeasts does not require warm temperatures, plenty of sunlight or carbon dioxide as algal cells do, and their cell walls are also relatively simple in comparison to indigestible algal cell walls [9]. In spite of this, the nucleic acid content of fungal and yeast cells (mainly available as RNA) remains higher than the recommended safe levels for human consumption (6–10%) and consequently is an important factor limiting nutritional value of SCP for animal or human consumption [2, 20]. A high level of nucleic acids in food is considered to be a problem as it can result in an increase serum uric acid levels that in turn results in kidney stone formation and gout [28]. To this end, various strategies have been successfully followed to reduce the nucleic acid content which include heat treatments, alkaline hydrolysis and enzymatic treatments [27]. Methods that have been developed to improve the digestibility of SCP products meanwhile, include mechanical disruption, autolysis and enzymatic treatment [6].

It is expected that dependence on SCP will increase as a result of increasing interest in healthy foods and successive food shortages. In support of this expectation, "Quorn" (a SCP produced from the mycelium of *Fusarium venenatus*) is now a FDA approved supermarket product in the European Union [46]. In addition, Torutein (a SCP produced by *Candida utilis*) is now used in Canada and Sweden as a highly nutritious flavor enhancer and replacement for meat, milk and egg protein [28].

The study aims to investigate the potential for using OP for production of SCP by *Aspergillus niger, Rhizopus oryzae* and *Saccharomyces cerevisiae* and to optimise the process parameters. In addition, a comparison of the amino acid profiles of SCP, produced to FAO standards, was made and means to reduce the nucleic acid content to recommended levels was tested. To the best of my knowledge, SCP production by *R. oryzae* is recorded for the first time here.

Materials and Methods

Microorganisms and preparation of orange peels and yeast extract

Aspergillus niger van Tiegh. 1867 [LEG; MB284309] and *Rhizopus oryzae* CBS 112.07 [Went & Prinsen Geerligs] were previously isolated from the peel surface of orange fruits and Egyptian soil respectively, identified by CBS-KNAW (Fungal Biodiversity Centre, Uppsalalaan, Netherlands), grown on potato-dextrose agar medium and routinely sub-cultured every other week. *Saccharomyces cerevisiae,* obtained from the Microbial Resource Centre, Faculty of Agriculture, Ain Shams University, was grown and routinely sub-cultured every other week on Sabouraud's medium.

Orange peels (OP) obtained from fresh mature navel orange fruits purchased from a local market were first air-dried and then oven dried at 55°C for 24 h until they reached a constant weight. The dried OP were then ground into 2–3 mm sized pieces and stored in dry flasks under dark conditions at room temperature.

Aliquots of 100 g of freshly harvested *S. cerevisiae* were mechanically disintegrated in Tris-HCl buffer (pH 9, at a solvent:biomass ratio of 14:1) followed by one of the following treatments: a- immediate lyophilisation (assigned as MD); b- heat shock by a sudden immersion of the flask containing the disintegrated biomass in a boiling water bath for 2 min, cooling in cold water, which was then followed either by immediate lyophilisation (assigned as HS0), or c- by incubation at 30°C for 6 h before lyophilisation (assigned as HS6); d- osmotic shock where a saturated solution of NaCl was immediately added to the disintegrated biomass and left for 30 min on a magnetic stirrer followed by lyophilisation (assigned as OS). Lyophilisation was performed at -65°C and 250 µbar using a Labconco freeze-dryer.

Fermentation media and optimisation of process parameters

Triplicate sets of 250 ml Erlenmeyer flasks, each containing the appropriate bed load of OP moistened to the appropriate level with either distilled water alone or distilled water mixed with other additives according to the experiment (sugars, molasses, yeast extracts, etc.), were sterilised by autoclaving at 121°C for 15 min. Autoclaving the OP results in a mild hydrolysis of carbohydrates, converting them into simpler forms, and causes the volatile oils that are known to inhibit the growth of microorganisms to evaporate. Initial pH of the orange peel medium (OPM) was adjusted before sterilisation to pH 5.5 using 0.1 M NaOH and HCl. Inoculation was performed using 1 ml of freshly prepared spore suspensions of *A. niger* (10^6 spores ml^{-1}), *R. oryzae* (10^6 spores ml^{-1}) or *S. cerevisiae* (3.5×10^4 spores ml^{-1}) which were previously prepared by adding 10 ml sterilised distilled water to 7-day old cultures. The spore loads used for *A. niger, R. oryzae* or *S. cerevisiae* were selected after preliminary experiments indicated the suitability of such ranges.

The strategy followed to optimise the important process parameters affecting the formation of biomass (BM), its protein content (PBM) and the total protein (TP), was to optimise each parameter independently with the optimal conditions for that parameter then being employed in subsequent experiments. The optimised parameters included initial moisture level (50–70% of the maximum water retention capacity, MWRC), bed loading (10–25 g OP), inoculum load (10^5–10^8 for *A. niger* and *R. oryzae* and 3.5–5.5×10^4 for *S. cerevisiae*), incubation period (48–168 h), agitation rate (125–225 rpm), initial pH of the fermentation medium (4.5–6.5), and incubation temperature (25–35°C). Supplementation with carbon sources was performed by addition of 1% w/w of sucrose, glucose, fructose, mannose, or molasses to the OPM while supplementation with different preparations of yeast extracts (YE) was through additions of 1% w/w. Concentrations of the optimum carbon source (1–3.5%, w/w) and YE preparation (1–3.5%, w/w) were also optimised.

Chemical analyses and determinations

Biomass (BM) of *A. niger*, *R. oryzae* and *S. cerevisiae* was determined according to the method described in Augustine *et al*. [4] so that triplicate samples of 1 g fermented substrate from each flask were transferred to centrifuge tubes containing 5 ml sodium sulfate (150 g l^{-1}) and centrifuged at 12,000 x*g* for 15 minutes to achieve complete separation of BM. At the end of centrifugation, the BM which floated (with a lower density than the substrate) was transferred to a pre-weighed filter paper and dried in hot air oven for 72 h at 85°C to obtain a constant weight.

Orange peels were analysed for cellulose, hemicellulose, pectin and lignin contents as described by Jermyn [18]. Total sugars were determined using the method of Dubois *et al*. [10] while glucose and fructose were determined by anthrone method [5]. Soluble, insoluble and the total nitrogen were estimated by the conventional micro-Kjeldahl method [22, 34]. MWRC (maximum amount of water absorbed) of the dried OP was determined using distilled water. The protein content of OP, BM and the fermentation residue was determined using bovine serum albumin as a standard [24]. Amino acid profiling of PBM was performed by hydrolysing 10 mg of the extracted protein with 6 M HCl at 110°C for 24 h in vacuum-sealed ampules, before final suspension in 1 mM HCl and analysis by HPLC (Knauar, C18 column).

Standardisation of the protein extraction method was achieved by testing the ability of various solutions at different solvent to biomass ratios (10:1–20:1) to extract the maximum amount of proteins from BM. One gram of BM was washed thrice in distilled water and frozen at -18°C for 6 h, followed by mechanical disintegration in one of the following buffers at different pH values 0.1 M citrate (pH 3–3.5), 0.2 M acetate (pH 4–5.5), 0.2 M phosphate (pH 6–7), 0.1 M Tris-HCl (pH 7–9) or 1 M sodium biocarbonate-sodiumcarbonate (pH 9.5–10), or in buffers with different concentrations (0.1–0.5 M) of NaCl or NaOH solution. The extract was subsequently centrifuged at 6000 rpm for 45 min and the supernatant was used for estimations of protein content. The protein yield from each solution (expressed as percent of the dry weight of the mycelium) was compared and the optimum conditions were applied throughout subsequent work. The temperature during the course of extraction was maintained at 4°C.

Nucleic acid content was extracted (with 0.5 M $HClO_4$), determined (at 260 nm) and reduced (by heating the biomass at different temperatures and different pH values for different times) as described by Ohta et al. [30].

Statistical validation of treatment effects

The mean, standard deviation, Tukey's test "T" and probability "P" values for three replicates of the investigated parameters and the control were calculated, and according to mathematical principles results were considered highly significant, significant or non-significant when $P < 0.01$, ≥ 0.01 and ≤ 0.05, and > 0.05 respectively [15].

Results and Discussion

Chemical analysis of OP, given in Table 1, revealed several advantages including a low level of lignin (0.2%, w/w) which makes the pre-treatments required for other agricultural wastes unnecessary [36]. Moreover, the availability of soluble sugars (32%, w/w) facilitates the germination of inoculated spores early in fermentation. Composition of OP, as in any natural product, is subject to change according to the orange variety, degree of ripening, methods and conditions of cultivation, storing, handling, and transport. Extraction of protein from the formed biomass was investigated and the highest protein yields were obtained from *A. niger* (27.0 mg %, w/w) and *R. oryzae* (22.0 mg %, w/w) with NaOH solutions of 0.4 M (at a ratio of 16:1, solvent:dried BM) and 0.3 M (at a ratio of 12:1) respectively. Tris-HCl buffer

(pH 9 at ratio of 14:1) meanwhile was the most efficient for *S. cerevisiae* (29 mg %, w/w) (Table 2). Higher ratios of extraction solution to dried BM did not yield increased amounts of protein, most probably due to all available protein having already been extracted (data not shown). Previous studies indicated that the maximum extraction of protein from *A. niger* was obtained with carbonate-bicarbonate buffer at pH 10 [3]. In contrast, protein extraction from *Chaetomium* spp. using phosphate buffer (pH 7) yielded the lowest amount of protein while its maximum amount was extracted with 1 M NaOH [47].

Table 1: Chemical composition and moisture level of the orange peels used as substrate.

Ingredient	Percent (%, w/w)
Carbohydrates	
Insoluble sugars	
Cellulose	17.3
Hemi-cellulose	13.5
Pectin	37.8
Lignin	0.2
Soluble sugars (SS)	32
Glucose	12.4
Fructose	10.5
Protein and nitrogen	
Soluble nitrogen	0.38
Insoluble nitrogen	1.64
Total nitrogen	2.02
Total protein	12.62
Maximum water retention capacity (MWRC)	35

Table 2: Extractability of protein from biomass using various solutions.

Extraction method[1] **(Mechanical disintegration followed by extraction with one of the following solutions):**		**Total protein extracted (mg / 100 g dry biomass)**[2]		
Solution	**Conc. /pH**	***A. niger*** (Ratio of 16:1)	***R. oryzae*** **(**Ratio of 12:1**)**	***S. cerevisiae*** **(**Ratio of 14:1**)**
NaOH	0.1 N	21.33 ± 0.45^{n}	$15.40 \pm 0.43^{*}$	$20.59 \pm 0.59^{*}$
	0.2 N	22.41 ± 0.31^{n}	$19.36 \pm 0.60^{*}$	$23.2 \pm 0.88^{*}$
	0.3 N	$26.19 \pm 0.71^{*}$	22.00 ± 0.63 •	$25.81 \pm 1.01^{*}$
	0.4 N	27.00 ± 0.81 •	22.00 ± 0.75^{n}	$26.39 \pm 0.89^{*}$
	0.5 N	27.00 ± 1.11^{n}	22.00 ± 0.83^{n}	$26.39 \pm 0.63^{*}$
NaCl	0.1 N	$7.67 \pm 0.27^{*}$	$6.82 \pm 0.22^{*}$	$15.08 \pm 0.57^{*}$
	0.2 N	$8.83 \pm 0.23^{*}$	$7.92 \pm 0.18^{*}$	$18.27 \pm 0.53^{*}$
	0.3 N	$10.72 \pm 0.17^{*}$	$9.02 \pm 0.31^{*}$	$19.14 \pm 0.66^{*}$
	0.4 N	$12.72 \pm 0.36^{*}$	$10.56 \pm 0.40^{*}$	$19.14 \pm 0.72^{*}$
	0.5 N	$12.855 \pm 0.23^{*}$	$10.56 \pm 0.62^{*}$	$19.14 \pm 0.57^{*}$
phosphate buffer	pH 6	$15.93 \pm 0.45^{*}$	$11.44 \pm 0.50^{*}$	$20.3 \pm 0.32^{*}$
	pH 7	$17.55 \pm 0.33^{*}$	$13.42 \pm 0.60^{*}$	$23.2 \pm 0.39^{*}$
Tris-HCl buffer	pH 8	$19.17 \pm 0.69^{*}$	$13.86 \pm 0.54^{*}$	$26.68 \pm 0.42^{*}$
	pH 9	$21.6 \pm 0.69^{*}$	$16.06 \pm 0.49^{*}$	29 ± 0.69 •
$NaHCO_3$- $Na_2(CO_3)$ buffer	pH 10	$21.6 \pm 0.51^{*}$	$16.06 \pm 0.62^{*}$	29 ± 0.72^{n}

1 Previous experiment was carried using NaCl and NaOH solutions and buffers at different ratios (10:1–20:1) and the best results were obtained with (0.4 M NaOH, 16:1), (0.3 M NaOH, 12:1), and (Tris-HCl, pH 9, 14:1)

for *A. niger*, *R. oryzae* and *S. cerevisiae* respectively. The given data are the amounts of protein extracted with different solutions and buffers at the optimum ratio for each organism.

2 Data (the average of three independent determinations approximated to one decimal point ± standard deviation) were statistically compared to the highest amount of protein extracted from each organism (•) where * = highly significant, ** = significant and n = non-significant.

The initial moisture level is a crucial factor that controls the activity of the microorganisms during SSF as most of the microbial growth takes place at or near the surface of the solid substrate. Maximum water retention capacity (MWRC) of the OP used in this study was found to be 35% w/w (Table 1). In the present study, the highest biomasses (BM) of *A. niger* (2.75 g/kg OP) and *R. oryzae* (2.28 g/kg OP) were attained with an initial moisture level of 60% with a protein content of the formed biomass (PBM) of 27% and 22.1% (w/w, grams of protein per 100 g BM) respectively, and total amounts of protein (TP) in the fermentation product (the formed BM plus the remaining OP) of 38% and 34% (w/w, grams of protein per 100 g fermentation product) respectively (Fig. 1). Meanwhile, a moisture level of 60% stimulated the highest BM (3.37 g/kg OP) of *S. cerevisiae* while the highest amount of PBM (29%, w/w) and TP (40.3%, w/w) were recorded at moisture level of 55% (Fig. 1). It is known that fungi have immense turgor pressure which assists in their penetration of hard substrates under conditions of low water availability, as seen in SSF [3]. In the present study, the optimum moisture level of OP found (55–60%, w/w) agrees with other research findings, which range from 40–70% [47]. Higher moisture levels lead to a decrease in substrate porosity, oxygen, heat and mass transfer, and cause particle agglomerations, thereby resulting in decreased microbial activity [31, 32]. On the other hand, lower moisture levels lead to poor accessibility of nutrients to microbial cultures resulting in poor microbial biomass yields [47].

The effect of varying the bed loading of flasks was also investigated and results indicated that a bed load of 20 g stimulated the maximum yield of BM (3.87, 3.42, 4.35 g/kg OP), PBM (27%, 24%, 31.32%; w/w) and TP (38.4%, 35.4%, 44.6%; w/w) for *A. niger*, *R. oryzae* and *S. cerevisiae* respectively (Fig. 2). Higher bed loading limited the levels of BM, PBM or TP produced by *A. niger*, *R. oryzae* and *S. cerevisiae,* this could be explained by a lower heat removal as the substrate load increases [11]. Investigations on the effect of varying the inoculum load on synthesis of BM, PBM and TP showed that the highest amounts of BM (5.09, 4.54, 5.29 g/kg OP), PBM (28.62%, 22.66%, 31.9%, w/w) and TP (39.7%, 34%, 44.90%, w/w) were obtained for *A. niger, R. oryzae* and *S. cerevisiae* respectively using inoculums containing 10^7 spores ml^{-1} for *A. niger* and *R. oryzae* and 5 x 10^3 spores ml^{-1} for *S. cerevisiae* (Fig. 3). In previous studies, maximum production of SCP was optimally obtained in *A. niger* and *Chaetomium* spp. using an inoculum load in the range of 10^6–10^8 spores ml^{-1} [7, 39, 47] while a load of 5.6 x 10^3 spore ml^{-1} was reported for yeasts [25]. In the present study, increase in spore count beyond the optimum led to a reduction in BM, PBM and TP, this is likely because competition among cells for disproportionate amounts of nutrients can induce autolysis [38]. Inoculation with lower loads of spores on the other hand, required a relatively longer time for cells to multiply to a sufficient number and produce the desired product.

Study of the time course of BM, PBM and TP production revealed that the maximum BM (6.32, 5.74, 5.69 g/kg OP) and TP (40.1%, 34.8%, 45.35%, w/w) amounts were obtained by *A. niger*, *R. oryzae* and *S. cerevisiae* respectively after 144 h (6 days) of incubation (Fig. 4). This agrees with previous research findings where the maximum yield of BM by *A. niger* and *Chaetomium* spp. was obtained on the 6^{th} day [47]. The maximal amount of PBM produced by *A. niger* (28.89%, w/w) was obtained after 120 h while there was no significant difference ($P > 0.05$) between the amounts of PBM synthesised by *R. oryzae* after 120 h.

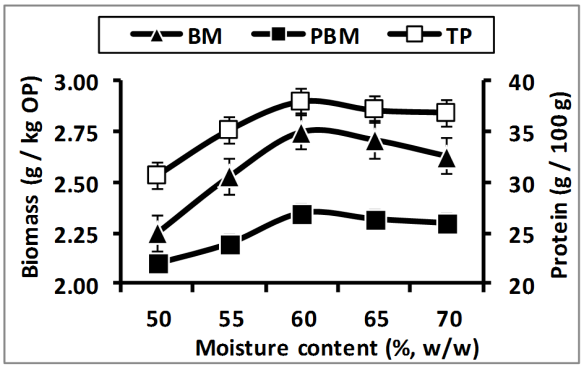

Fig. 1-a: *Aspergillus niger*

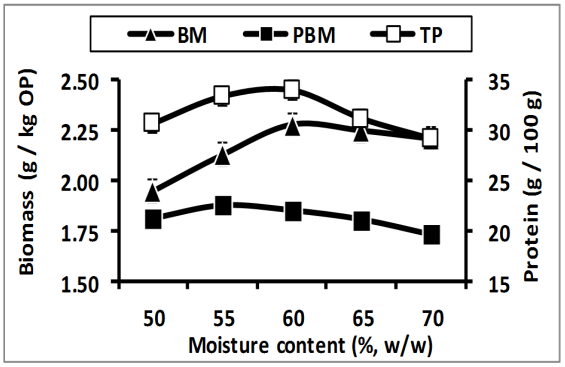

Fig. 1-b: *Rhizopus oryzae*

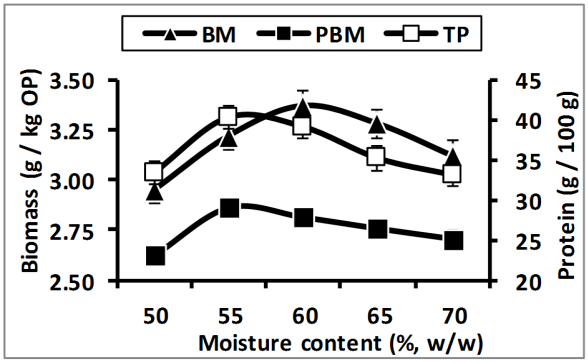

Fig. 1-c: *Saccharomyces cerevisiae*

Fig. 1: Effect of initial moisture content (%, w/w) on production of biomass (BM, –▲–), protein content of the formed biomass (PBM, –■–) and the total protein content of the fermentation product (TP, –□–).
Data are the average of three independent determinations ± standard error.

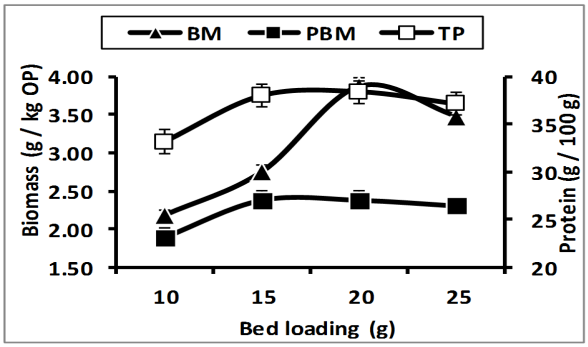

Fig. 2-a: *A. niger*

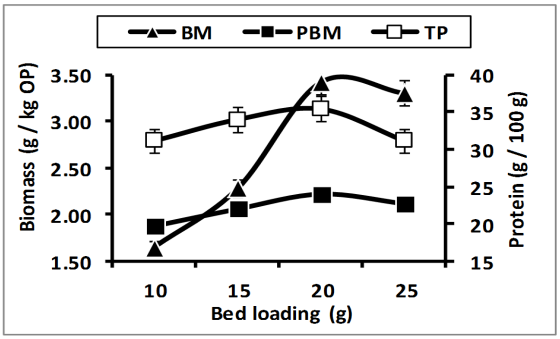

Fig. 2-b: *R. oryzae*

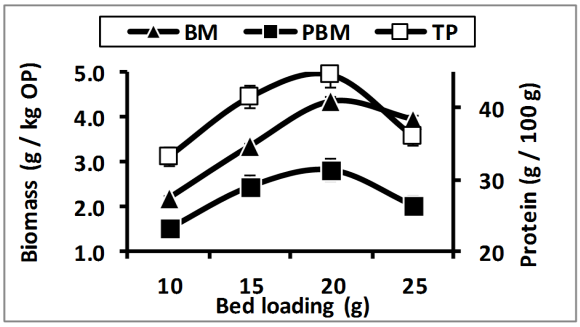

Fig. 2-c: *S. cerevisiae*

Fig. 2: Effect of bed loading (g) on production of biomass (BM, –▲–), protein content of the formed biomass (PBM, –■–) and the total protein content of the fermentation product (TP, –□–).
Data are the average of three independent determinations ± standard error.

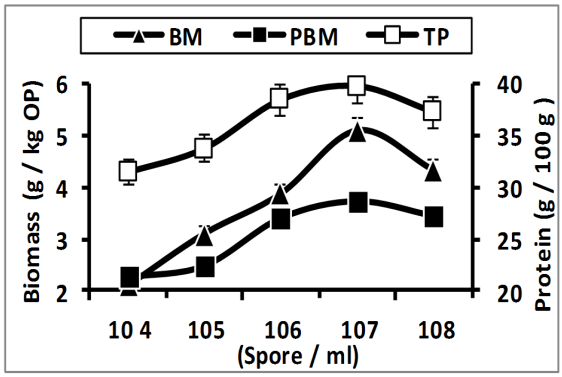

Fig. 3-a: *A. niger*

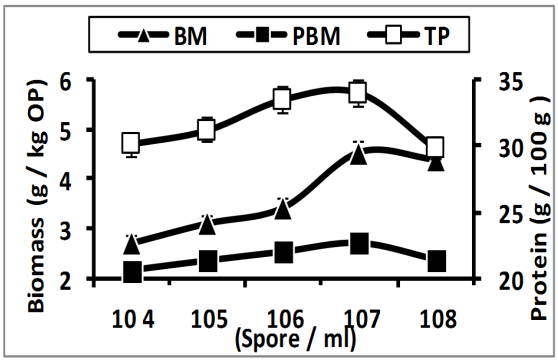

Fig. 3-b: *R. oryzae*

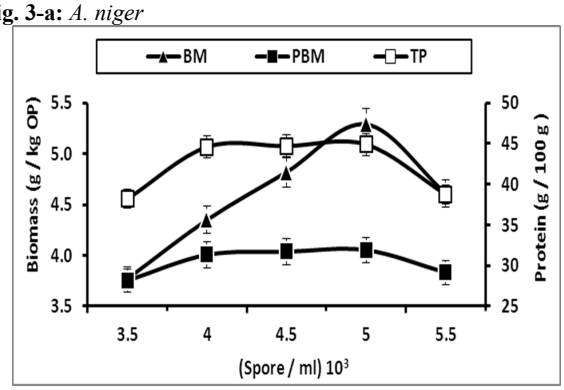

Fig. 3-c: *S. cerevisiae*

Fig. 3: Effect of varying the spore load (spores ml^{-1}) of inoculum on production of biomass (BM, –▲–), protein content of the formed biomass (PBM, –■–) and the total protein content of the fermentation product (TP, –□–).
Data are the average of three independent determinations ± standard error.

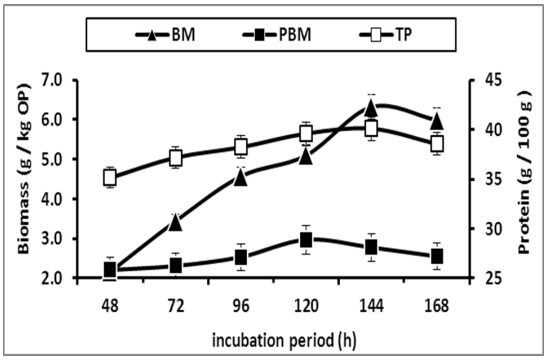

Fig. 4-a: *A. niger*

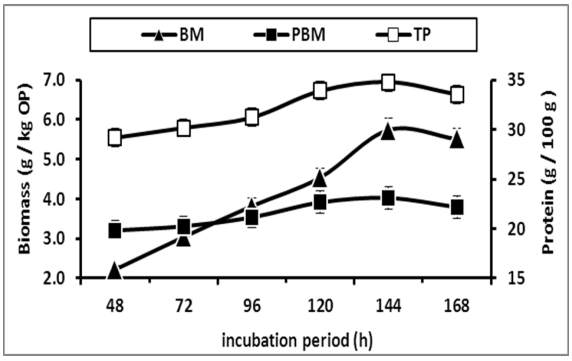

Fig. 4-b: *R. oryzae*

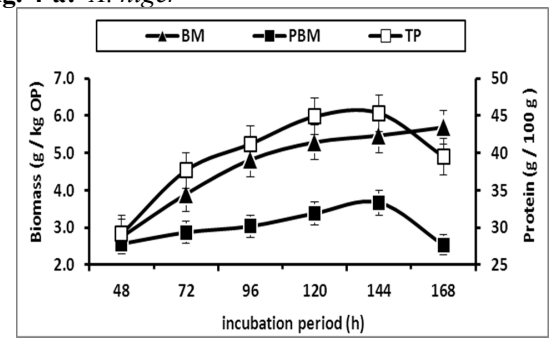

Fig. 4-c: *S. cerevisiae*

Fig. 4: Effect of various incubation periods (h) on production of biomass (BM, –▲–), protein content of the formed biomass (PBM, –■–) and the total protein content of the fermentation product (TP, –□–).
Data are the average of three independent determinations ± standard error.

(22.85%, w/w) and 144 h (22.9%, w/w). Meanwhile, PBM of *S. cerevisiae* (33.35%, w/w) was optimally obtained after 144 h (Fig. 4). Indeed, previous work has indicated that the optimum incubation periods for BM and PBM are not necessarily synchronised. For example, maximum BM synthesis of *S. cerevisiae* and *C. tropicalis* was observed after 7 days of incubation while the maximal amount of PBM was obtained on the 3rd day [9]. Moreover, production of PBM achieved a maximum in *A. niger* and *Chaetomium* spp. after 4 and 5 days of incubation respectively, versus 6 days for the maximum synthesis of BM [47]. In the present study, PBM and TP produced by *A. niger, R. oryzae* and *S. cerevisiae*, gradually decreased after the optimum incubation period which could be attributed to the depletion of nutrients and release of waste products from the cells [9].

Investigations into the effect of agitation rate revealed that the maximum amounts of BM and PBM produced by *A. niger* (7.26 g/kg OP; 28.89%, w/w) and *R. oryzae* (6.18 g/kg OP; 23.76%, w/w) respectively, were stimulated at 175 rpm (Fig. 5-a, b). On the other hand, the curves representing the relationship between the agitation rate within the range of 150–175 rpm and production of BM, PBM and TP by *S. cerevisiae* plateau, so that the amounts produced at 150 rpm (5.69 g/kg OP; 33.35%, w/w; 45.35%, w/w), 175 rpm (5.89 g/kg OP; 33.54%, w/w; 45.7%, w/w) and 200 rpm (6.03 g/kg OP; 33.93%, w/w; 45.81%, w/w) respectively, were not significantly different ($P > 0.05$) (Fig. 5-c). Enhancement in BM, PBM and TP amounts due to increasing the agitation rate to 175 rpm could be explained by the simultaneous enhancement in aeration and heat transfer, while the decline in BM, PBM and TP amounts at higher agitation rates could be explained by an increase the shear forces which can lead to an increase in the vacuolation and/or accelerated fragmentation of hyphae [45]. Indeed, it was also observed in another study that the mechanical agitation in SSF did not show a pronounced effect in all cases tested [26].

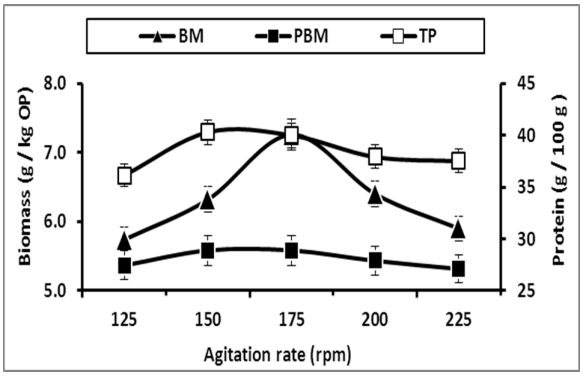

Fig. 5-a: *A. niger*

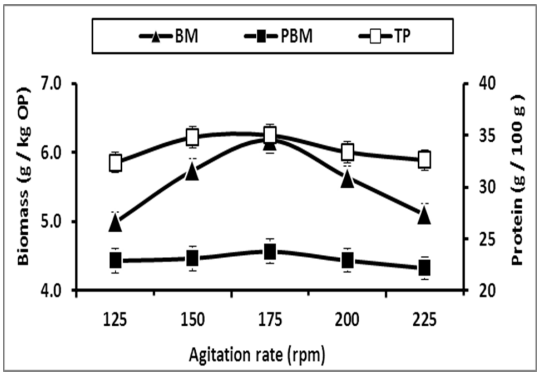

Fig. 5-b: *R. oryzae*

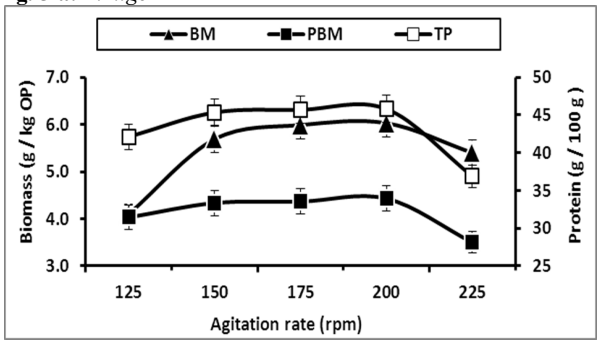

Fig. 5-c: *S. cerevisiae*

Fig. 5: Effect of varying the agitation rate (rpm) on production of biomass (BM, –▲–), protein content of the formed biomass (PBM, –■–) and the total protein content of the fermentation product (TP, –□–).
Data are the average of three independent determinations ± standard error.

The influence of varying the initial pH of the medium on formation of BM, PBM and TP was investigated with the results demonstrating a significant increase in the amounts produced by *A. niger* (8.55 g/kg OP; 29.97%, w/w; 40.91%, w/w), *R. oryzae* (6.86 g/kg OP; 25.96%, w/w; 37.36%, w/w) and *S. cerevisiae* (6.67 g/kg OP; 35.09%, w/w; 46.52%, w/w) respectively, when the initial pH increased from 5.5 (the original initial pH of the medium) to pH 6 (Fig. 6). Statistical analysis on the amounts of TP produced by *S. cerevisiae* at pH 6 (46.52%, w/w) and pH 5.5 (45.8%, w/w) showed the difference was not significant ($P > 0.05$). These results agree with the known acidophilic nature of most yeasts and filamentous fungi, which has the advantage of preventing bacterial contamination in long-term cultures [46], and coincides with the findings of other studies where acidic pH values, e.g. pH 4 and 5.5, were optimal for SCP production by *Penicillium expansum*, [19] and *Chaetomium* spp. [47]. A neutral pH (pH 7), on the other hand, was shown as the optimum for SCP production by *A. niger* [47].

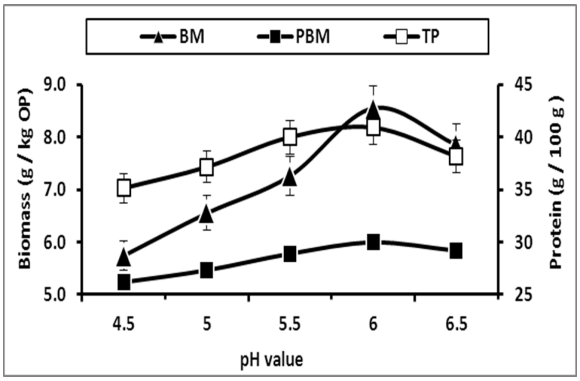

Fig. 6-a: *A. niger*

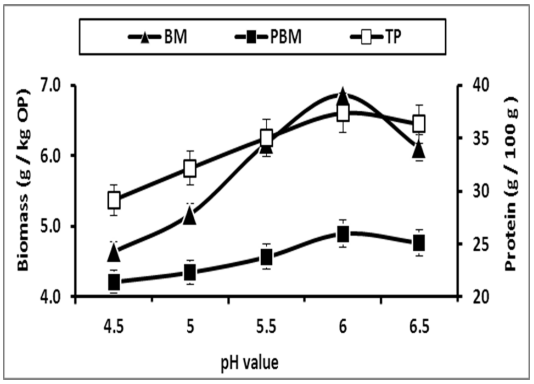

Fig. 6-b: *R. oryzae*

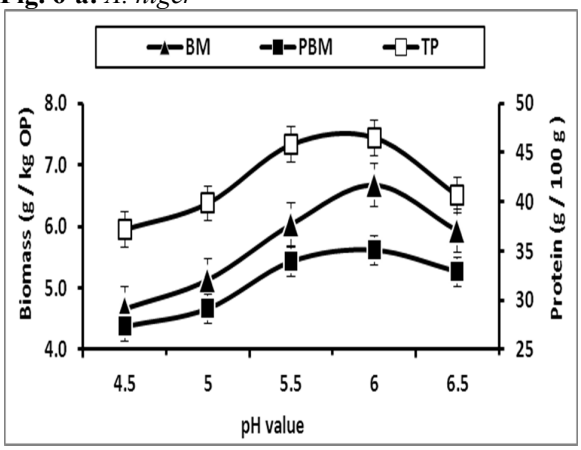

Fig. 6-c: *S. cerevisiae*

Fig. 6: Effect of varying the initial pH value of the fermentation medium on production of biomass (BM, –▲–), protein content of the formed biomass (PBM, –■–) and the total protein content of the fermentation product (TP, –□–).
Data are the average of three independent determinations ± standard error.

Investigating the effect of varying the incubation temperature showed that BM, PBM and TP were maximally produced by *A. niger* (9.16 g/kg OP; 30.24% w/w; 41.24% w/w), *R. oryzae* (6.86 g/kg OP; 25.96%, w/w; 37.36%, w/w) and *S. cerevisiae* (6.7 g/kg OP; 35.09%, w/w; 46.52%, w/w) respectively, at 27.5°C (Fig. 7). At lower temperatures, the decline in protein content may be due to inactivation of cellular activities while at higher temperatures the enzymes of the cell may be denatured [42]. Temperature also affects the metabolic activities associated with substrate oxidation and oxygen diffusion, which both affect growth rate and biomass synthesis [46].

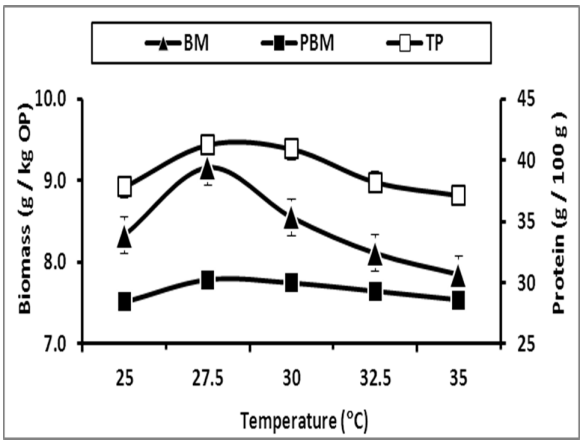

Fig. 7-a: *A. niger*

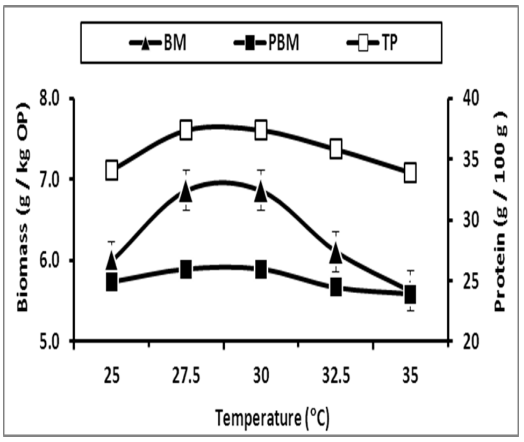

Fig. 7-b: *R. oryzae*

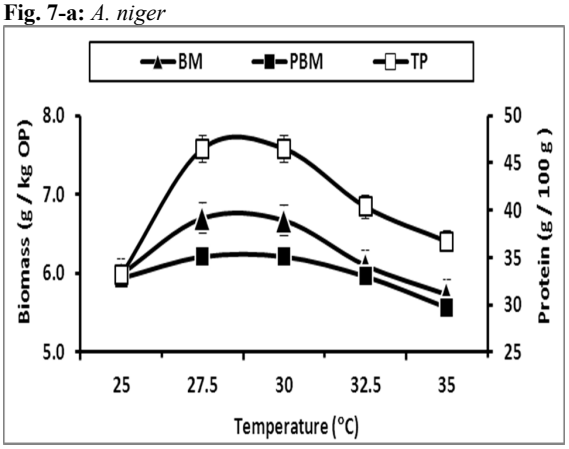

Fig. 7-c: *S. cerevisiae*

Fig. 7: Effect of varying the temperature (°C) of incubation on production of biomass (BM, –▲–), protein content of the formed biomass (PBM, –■–) and the total protein content of the fermentation product (TP, –□–).
Data are the average of three independent determinations ± standard error.

Selecting a suitable carbon source, and optimising its concentration, is of prime importance in microbial fermentations where around 50% of the carbon source supplied in the medium is assimilated to build cellular materials [35]. In an attempt to overcome the public resistance shown towards consuming SCP and mini-foods, simple pure sugars (sucrose, glucose, fructose and mannose) and molasses were selected to investigate their potential to enrich the OPM as they are of natural origin and are already consumed as part of human diets. The results showed that, with the exception of mannose which resulted in a significant reduction in the BM formed (9 g/kg OP) and TP produced (39.7%, w/w) by *A. niger*, and PBM (26%, w/w) and TP (36.11%, w/w) produced by *R. oryzae*, all other tested sugars resulted in a significant increase in BM, PBM and TP production (Fig. 8). Maximum yields of BM, PBM and TP were produced by *A. niger* (9.38 g/kg OP; 35.1%, w/w; 45.2%, w/w; Fig. 8-a) and *R. oryzae* (7.34 g/kg OP; 26.84%, w/w; 37.84%, w/w; Fig. 8-b) in the presence of 1% w/w sucrose, and for *S. cerevisiae* in the presence of 1% w/w glucose (9.46 g/kg OP; 40.89%, w/w; 50.89%, w/w; Fig. 8-c). Other studies meanwhile have shown that supplementing banana peel extract with sucrose, glucose, maltose, in that order, resulted in the highest production of BM and PBM by *Trichoderma harzianum* [41] while supplementing various agricultural wastes with sucrose, glucose, and galactose, in that order, stimulated production of BM and PBM by *P. expansum* [19]. Moreover, previous work showed that *S. cerevisiae* was optimally grown on molasses for production of SCP [23]. The hydrolytic enzymes of *A. niger, R. oryzae* and *S. cerevisiae*, specifically cellulases and pectinases, play an important role in making more sugars available in the medium [3, 16] and indeed our

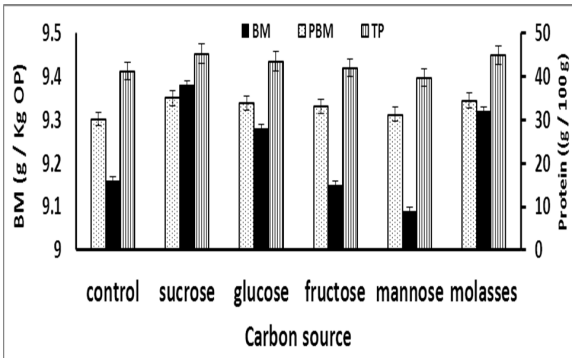

Fig. 8-a: *A. niger*

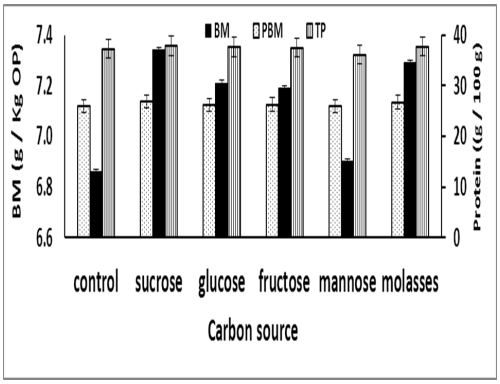

Fig. 8-b: *R. oryzae*

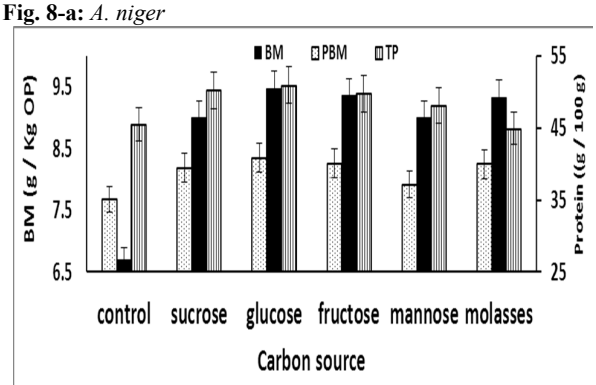

Fig. 8-c: *S. cerevisiae*

Fig. 8: Effect of adding 1% (w/w) of various sugars to the fermentation medium on production of biomass (BM), protein content of the formed biomass (PBM) and the total protein content of the fermentation product (TP).
Data are the average of three independent determinations ± standard errors.

analysis of OP revealed the presence of considerable amounts of cellulose (17.3%, w/w) and pectin (37.8%, w/w) in this substance (Table 1). Further optimisation was performed by investigating the effect of various concentrations of sucrose and glucose on production of BM, PBM and TP. The results demonstrated that the maximum amounts of BM (12.27, 9.35, 11.39 g/kg OP), PBM (37.26%, 28.6%, 41.76%; w/w) and TP (48.56%, 37.9%, 53.16%; w/w) were produced by *A. niger* and *R. oryzae* at 2% and 1.5% w/w sucrose respectively (Fig. 9-a, b), and by *S. cerevisiae* at 2% w/w glucose (Fig. 9-c).

In spite of the superiority of organic nitrogen sources in stimulating the formation of many microbial products, including SCP, inorganic sources are still generally preferred due to their relative low cost. However, if the SCP product contains many synthetic ingredients, this will increase resistance to the acceptance of this product. Therefore, different preparations of *S. cerevisiae* (YE) produced in this work were used to supplement the cultures of *A. niger*, *R. oryzae* and *S. cerevisiae* grown on OP supplemented with sucrose or glucose. Comparing the highest amounts of BM (17.32, 12.77, 14.44 g/kg OP), PBM (43.74%, 36.96%, 43.21%; w/w) and TP (53.2%, 47.69%, 54.28%; w/w) produced by *A. niger, R. oryzae* and *S. cerevisiae* respectively, in presence of YE (MD; as described in materials and methods) with the corresponding amounts of BM (17.32, 12.1, 13.9 g/kg OP), PBM (43.98%, 36.9%, 43%; w/w), and TP (53.2%, 47%, 53.8%; w/w) produced in presence of YE (Difco) indicated a non-significant, significant and highly significant difference for each measure of yield respectively (Fig. 10). The lowest amounts of BM, PBM and TP were obtained in the presence of YE prepared by osmotic shock (OS), but these were still significantly higher than the corresponding amounts produced in complete absence of YE (Fig. 10). The stimulating

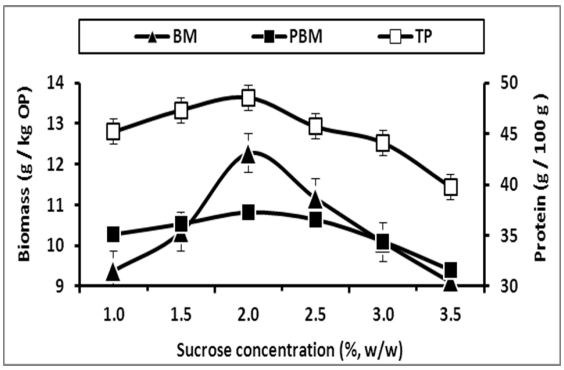

Fig. 9-a: *A. niger*

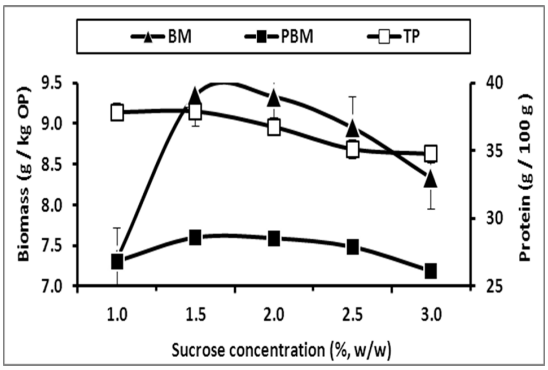

Fig. 9-b: *R. oryzae*

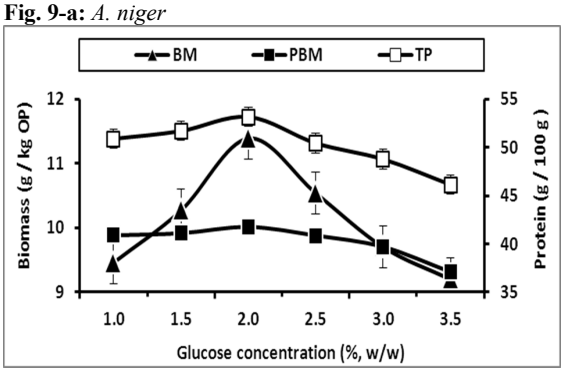

Fig. 9-c: *S. cerevisiae*

Fig. 9: Effect of varying the concentration of sucrose and glucose on production of biomass (BM, –▲–), protein content of the formed biomass (PBM, –■–) and the total protein content of the fermentation product (TP, –□–). Data are the average of three independent determinations ± standard error.

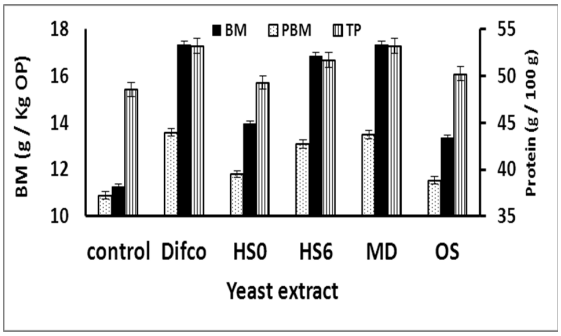

Fig. 10-a: *A. niger*

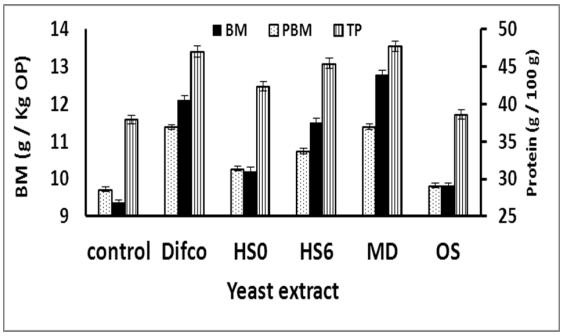

Fig. 10-b: *R. oryzae*

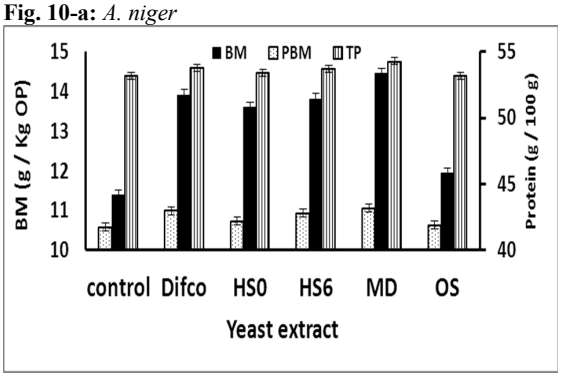

Fig. 10-c: *S. cerevisiae*

Fig. 10: Effect of adding 1% (w/w) yeast extract prepared by different methods on production of biomass (BM), protein content of the formed biomass (PBM) and the total protein content of the fermentation product (TP). Data are the average of three independent determinations ± standard error.

effect of YE could be ascribed to its peptide, amino acid, and water-soluble vitamin (such as B_1, B_2, B_6) contents, in addition to the presence of some growth factors which support microbial growth [17]. An enhancement effect of inorganic nitrogen sources such as $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$, and a mixture of $(NH_4)_2SO_4$ and urea, on production of SCP by *A. niger*, *Chaetomium* spp., *Fusarium graminearum*, *Penicillium janthinellum Trichoderma harzianum* and *S. cerevisiae,* was previously reported [28, 36, 41, 47]. Increasing the concentration of the supplemented YE (MD) from 1% to 2.5% (w/w) resulted in a significant increase in BM (18.59, 14.98, 16.65 g/kg OP), PBM (45.9%, 40.26%, 44.08%; w/w) and TP (56.14%, 51.1%, 56.11%; w/w) produced by *A. niger*, *R. oryzae* and *S. cerevisiae* respectively (Fig. 11).

The amounts of BM, PBM and TP obtained after optimising the physico-chemical parameters (Fig. 11) represent 6.76, 6.57, and 4.94-fold increase in BM; 1.7, 1.8, and 1.52-fold increases in PBM; and 1.47, 1.50, and 1.39-fold increases in TP when compared to the initial amounts of BM (2.75, 2.28, 3.37 g/kg OP), PBM (27%, 22.1%, 29%; w/w), TP (38%, 34%, 40.3%; w/w) produced by *A. niger, R. oryzae* and *S. cerevisiae* respectively, as shown in Fig. 1. The levels of PBM and TP obtained from the dry BM of *A. niger*, *R. oryzae* and *S. cerevisiae*, recommends these products as potential food sources and feed supplements when they are compared to the PBM produced by other microorganisms, e.g. 18–22% from *Polyporus* [29], 20.6% from *Aspergillus terreus* [13], 22.6% from *Pleurotus ostreatus* [40], 40–50% from *Penicillium janthinellum* [36], 43% from *Aspergillus oryzae* [37] and up to 55% from *Paecilomyces variotti* [28].

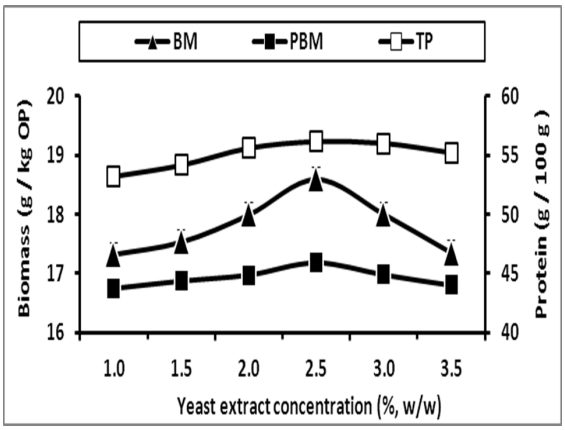

Fig. 11-a: *A. niger*

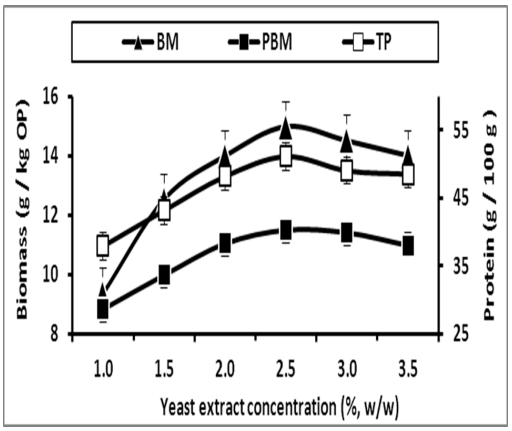

Fig. 11-b: *R. oryzae*

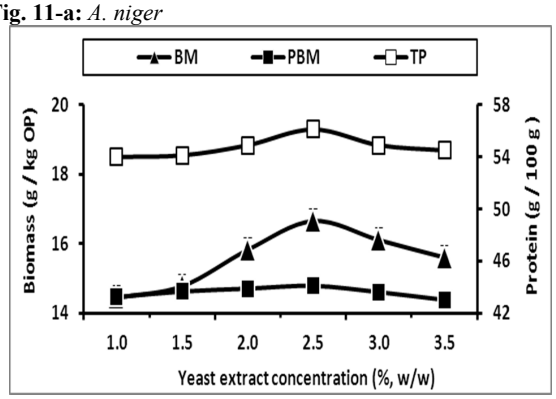

Fig. 11-c: *S. cerevisiae*

Fig. 11: Effect of various concentrations (%, w/w) of yeast extract on production of biomass (BM, –▲–), protein content of the formed biomass (PBM, –■–) and the total protein content of the fermentation product (TP, –□–).
Amount of TP represent the amount of protein detected in the fermentation product minus the amount of protein detected in the yeast extract supplemented to the OPM. Data are the average of three independent determinations ± standard error.

In addition to growth yield and percentage of protein, amino acid profiles and nucleic acid content are important criteria in evaluating the nutritional value of a SCP [28].

Therefore, the amino acid profiles of PBM produced by *A. niger, R. oryzae* and *S. cerevisiae*, were determined and data indicated that they compare favourably with FAO standards (Table 3). Amino acid contents of SCP produced in other studies by *A. niger*, *Trichoderma reesei*, *Kluyveromyces marxianus* and *P. janthinellum* also compared well with FAO standards [14, 21, 36]. However, as shown in Table 3, the nucleic acid contents of PBM produced by *A. niger* (8.9%, w/w), *R. oryzae* (9.4% w/w) and *S. cerevisiae* (8.4% w/w) are almost five times higher than the acceptable levels for human consumption [46]. Therefore, the effects of heat treatment at various temperatures (50–100°C) and pH values (2–10) for different periods of time (10–30 min) on the nucleic acid content of BM of *A. niger, R. oryzae* and *S. cerevisiae*, were investigated and the results of the successful treatments are given in Table 4. It is clear that heat treatment at 90°C for 20 min at pH 9, significantly reduced the nucleic acid content of the BM to 1.7%, 1.9% and 1.4% for each species respectively, the values of which all lie within the accepted levels recommended by the FAO. It was also observed that heating the BM of *R. oryzae* for 20 min at 90°C and pH 2 resulted in a reduction of the nucleic acid content to the same level obtained at pH 9 (1.9%, w/w). Neither increasing the exposure time from 20 to 25 min nor the temperature from 90 to 100°C resulted in a more significant decrease ($P > 0.05$) (Table 4). In addition to thermal treatments, treatments with NaOH and NaCl, activation of endogenous nucleases, and use of pancreatic ribonucleases were all previously proven to reduce the nucleic acid content of *Candida lipolytica, C. utilis* and *Rhodotorula glutinis* to 1% [1, 28, 43, 49]. Subsequent specialised treatments on the products may be performed to obtain SCP with certain characteristics, such as modifications in the levels of vitamins, nucleic acids, amino acid etc., for use in specific applications.

Table 3: Amino acid profiles and nucleic acid contents of PBM produced by *A. niger, R. oryzae* and *S. cerevisiae* as compared to FAO standards.

Composition (g 100 g PBM^{-1})	*A. niger*	*R. oryzae*	*S. cerevisiae*	FAO standards*
Amino acid				
Cystine + methionine	3.4 ± 0.04	3.7 ± 0.12	2.5 ± 0.08	2.2
Leucine	1.9 ± 0.04	2.4 ± 0.06	2.3 ± 0.06	2.2
Lysine	9.4 ± 0.16	8.6 ± 0.14	7.3 ± 0.14	1.6
Methionine	2.7 ± 0.15	2.9 ± 0.09	1.9 ± 0.05	2.2
Phenylalanine	2.9 ± 0.07	2.6 ± 0.07	3.1 ± 0.12	2.2
Threonine	3.2 ± 0.09	3.4 ± 0.07	3.7 ± 0.10	1.0
Tryptophan	0.9 ± 0.03	0.8 ± 0.01	1.1 ± 0.04	0.5
Tyrosine	3.1 ± 0.05	2.9 ± 0.06	3.7 ± 0.10	2.8
Valine	6.2 ± 0.16	5.1 ± 0.14	4.8 ± 0.08	1.6
Nucleic acid	8.9 ± 0.32	9.4 ± 0.26	8.4 ± 0.26	2

*http://www.fao.org.

Concluding remarks

In conclusion, the potential for the use of OP, a cheap and inexpensive agricultural waste, to produce high yields of BM, PBM and TP by *A. niger, R. oryzae* and *S. cerevisiae* was demonstrated. SCP of *A. niger* and *S. cerevisiae* have the advantages of being synthesised on a combination of OP, sucrose and glucose which are natural products already used as foods. Moreover, these organisms are classified as GRAS and their use in various food applications are well established. Production of SCP by *R. oryzae*, to the best of my knowledge, has not been previously reported. The amino acid profiles and the nucleic acid contents (after reduction) of the produced SCP was comparable to FAO standards, therefore advocating their use in food applications. It is however, recommended that further large-scale studies be carried out in addition to extensive toxicological and acceptability tests.

Table 4: Effect of pH-temperature-time relationship on nucleic acid content of *A. niger. R. oryzae* and *S. cerevisiae*.

pH value	Temp. (°C)	Nucleic acid content (%, w/w)								
		A. niger			*R. oryzae*			*S. cerevisiae*		
		Time (min)			Time (min)			Time (min)		
		15	20	25	15	20	25	15	20	25
Untreated		8.9 ± 0.32			9.4 ± 0.26			8.4 ± 0.26		
2	**80**	3.1 ± 0.09	2.9 ± 0.06	2.9 ± 0.08	2.4 ± 0.07	1.9 ± 0.08	1.9 ± 0.06	3.8 ± 0.11	3.2 ± 0.10	3.0 ± 0.05
	90	2.9 ± 0.08	2.9 ± 0.08	2.8 ± 0.07	2.3 ± 0.05	1.9 ± 0.05	1.8 ± 0.05	3.1 ± 0.07	2.5 ± 0.07	2.5 ± 0.09
	100	2.9 ± 0.11	2.8 ± 0.11	2.8 ± 0.08	2.3 ± 0.06	1.8 ± 0.04	1.8 ± 0.05	2.8 ± 0.11	2.5 ± 0.04	2.4 ± 0.06
3	**80**	4.2 ± 0.07	4.0 ± 0.09	3.8 ± 0.12	4.0 ± 0.14	3.3 ± 0.11	3.2 ± 0.10	4.7 ± 0.12	4.3 ± 0.13	4.1 ± 0.16
	90	3.9 ± 0.07	3.6 ± 0.12	3.5 ± 0.11	3.7 ± 0.11	3.2 ± 0.13	2.9 ± 0.08	4.6 ± 0.16	4.2 ± 0.12	4.1 ± 0.13
	100	3.7 ± 0.09	3.5 ± 0.07	3.2 ± 0.12	3.6 ± 0.13	3.0 ± 0.09	2.9 ± 0.08	4.4 ± 0.12	4.1 ± 0.14	3.9 ± 0.11
9	**80**	4.0 ± 0.09	2.6 ± 0.12	2.5 ± 0.06	2.5 ± 0.07	2.0 ± 0.08	1.9 ± 0.05	2.8 ± 0.08	2.1 ± 0.06	2.0 ± 0.08
	90	3.2 ± 0.08	1.7 ± 0.08	1.7 ± 0.02	2.4 ± 0.06	1.9 ± 0.04	1.8 ± 0.05	2.0 ± 0.06	1.4 ± 0.03	1.4 ± 0.06
	100	3.1 ± 0.10	1.7 ± 0.04	1.7 ± 0.03	2.4 ± 0.04	1.9 ± 0.06	1.8 ± 0.04	1.9 ± 0.04	1.3 ± 0.04	1.3 ± 0.03
10	**80**	3.9 ± 0.11	2.7 ± 0.02	2.6 ± 0.08	3.2 ± 0.09	2.0 ± 0.04	1.9 ± 0.07	3.8 ± 0.11	3.4 ± 0.08	3.0 ± 0.12
	90	3.4 ± 0.09	2.6 ± 0.06	2.6 ± 0.10	2.5 ± 0.06	1.9 ± 0.07	1.9 ± 0.05	3.3 ± 0.10	2.9 ± 0.11	2.8 ± 0.09
	100	3.3 ± 0.10	2.5 ± 0.05	2.5 ± 0.07	2.4 ± 0.09	1.9 ± 0.05	1.9 ± 0.04	3.3 ± 0.13	2.8 ± 0.07	2.8 ± 0.06

Data are the average of three independent determinations approximated to one decimal point ± standard deviation.

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