Research article

Investigation of the Yeast *Yarrowia lipolytica* Cultivation on Tuna Head Protein Hydrolysate

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Curr. Appl. Sci. Technol. Year 2024, Vol. 24 (No. 3), e0258799; https://doi.org/10.55003/cast.2023.258799

Received: 15 May 2023, Revised: 23 June 2023, Accepted: 29 September 2023, Published: 18 December 2023

Keywords

fish by-product;
fish protein hydrolysate;
Thunnus albacares;
Yarrowia lipolytica;
yeast cultivation;
yellowfin tuna heads

Abstract

Tuna (*Thunnus* spp.) is a significant commodity within the fishing industry. However, up to half of the total tuna weight ends up as waste, including tuna heads, which contain valuable nutrients such as protein, polyunsaturated fatty acids, enzymes, minerals, and trace elements. The purpose of this study was to investigate an environmentally sustainable approach for tuna head processing by yeast cultivation. The oleaginous yeast *Yarrowia lipolytica* was successfully cultured on tuna head protein hydrolysate and the effects of culture conditions including inoculum size, hydrolysate pH and incubation volume on nutrition utilization and on yeast growth were investigated. *Y. lipolytica* showed good biomass accumulation when cultured on tuna head protein hydrolysates. The yeast biomass increased with decreasing incubation volume and reached 8.6 g/L. The yeast also showed the ability to reduce up to 83.7% of hydrolysate lipid content while remaining 70.7% of nitrogen content. The culture conditions had more effect on lipid reduction than on nitrogen reduction and a good correlation between the specific growth rate and lipid reduction was observed. The yeast biomass was 64.0% in protein content and 9.6% in lipid content, without optimization. The investigation results demonstrated the potential improvement of tuna head utilization as well as the potential application of yeast biomass cultivation.

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1. Introduction

Tuna (*Thunnus* spp.) has been recognized as a significant commodity within the fishing industry. In 2018, the global catch of tuna was estimated to be around 5.5 million tons, with an estimated value of USD 40 billion [1]. Approximately, half of the total tuna weight ends up as waste, and if not properly treated, could lead to severe water pollution that negatively impacts the fishing industry [2]. To support sustainable fishing practice, it is crucial to have proper disposal methods for tuna waste.

The majority of tuna waste is tuna heads (34% w/w) [3]. These tuna heads were found to contain many polyunsaturated fatty acids (eicosapentaenoic (EPA) and docosahexaenoic (DHA)), proteins and enzymes, minerals, and trace elements [4]. These valuable nutrients can be extracted by hydrolyzation, resulting in tuna oil and protein hydrolysate. Cultivating microorganisms with tuna head hydrolysate is a low-input approach compared to conventional methods such as fish meal processing [5]. Yeast cells are useful for production of value-added products or microbial biomass [6]. Traditionally, yeast biomass production relies on many agricultural materials such as corn and manioc starch [7]. However, using food crops can affect food security and reduce the sustainability of yeast biomass production [8]. Therefore, many studies on yeast have focused on the potential of using food processing by-products such as fishery waste hydrolysate for cultivation of yeast. Ferrer *et al*. [9] successfully cultured *Saccharomyces cerevisiae* biomass using acid hydrolysates of shrimp-shell waste, demonstrating the possibility of using aquaculture by-products in biomass production. Zeng *et al*. [10] reported the potential to culture the oleaginous yeast *Rhodotorula toruloides* on food waste biomass hydrolysate for biolipids and single cell protein production. Fish sources used for yeast biomass production include cod viscera, tuna stomach, salmon by-products, silver carp by-products or small fish [6, 11]. To the best of our knowledge, tuna heads have not been previously used for yeast biomass production.

The oleaginous yeast *Y. lipolytica* has been well-studied in many industrial applications such as the production of polyunsaturated fatty acids (PUFAs), single-cell protein, citric acid and carotenoids [12]. This yeast is known to possess numerous desired features for industrial settings such as safety and robustness, growth at highly dense cell density and ease of genetic modification. The utilization of tuna head hydrolysate in *Y. lipolytica* cultivation can serve the dual purpose of promoting sustainability for the fishery industry and providing a low-cost feedstock that can be utilized in the production of microbial-based value-added products.

The objective of this study was to investigate the cultivation of the oleaginous yeast *Y. lipolytica* in the hydrolysate from tuna heads. Tuna head protein hydrolysates (TPH) were obtained with Protamex, a commercial protease, and was used for yeast cultivation. Yeast growth was evaluated through biomass accumulation and specific growth rate. The TPH protein and lipid reduction were also investigated.

2. Materials and Methods

2.1 Tuna head and yeast strains

The yellowfin tuna heads were purchased from the JK Fish Company Ltd. (Nha Trang City, Khánh Hòa Province, Vietnam). On average, each head weighed approximately 5 kg. The tuna was caught using a handline or longline method. The heads were washed with tap water, ground to a suitable size (~3 mm plate) using a meat grinder (TA57D, Code 947000, Andritz Frautech S.r.l., Schio, Vicenza, Italy) and then divided into portions of 100 g and stored in PE plastic bags at -20°C prior to use in less than 6 months.

The yeast strain *Y. lipolytica* VTCC 0544 obtained from the Vietnam Type Culture Collection (Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi, Vietnam) was used throughout the project. The yeast was stored in 15% glycerol at -80°C in 1.8 mL sterile cryotube vials (Thermo Fisher Scientific, Roskilde, Denmark).

2.2 Preparation of protein hydrolysate from tuna head

The ground heads were thawed at room temperature (30±2°C) and hydrolyzed using the enzyme Protamex (*Bacillus* protease) with a declared activity of 1.5 Anson Unit/g (Novozyme, Bagsvaerd, Denmark). The ratio of enzyme to substrate was 0.5% w/w, water: raw material was 1:1 w/w with a natural pH of ~6.5. The mixture was incubated at 55°C for 4 h. The hydrolysis was stopped by heating the mixture to 95°C for 1 h. Liquefied tuna heads were filtered using a household wire mesh beverage sieve and centrifuged (MF600, Daihan Labentech, Hwado-eup, Namyangju-si, Gyeonggi-do, Korea) at 2820 x g (3500 rpm) for 10 min at room temperature. The supernatant, which contained most of the lipids, was then separated from the protein hydrolysate by removing 10% of the liquid (i.e., for each 400 mL centrifuged hydrolysate, 40 mL of supernatant that contains lipids was removed). The protein hydrolysate was autoclaved (121°C, 20 min) and then inoculated with the yeast.

2.3 Inoculation of *Y. lipolytica* on tuna head protein hydrolysate

The yeast was activated for 48 h at 27°C using medium YPDA (10% yeast extract, 20% fish peptone, 20% glucose (dextrose), 15% agar-agar) (Biobasic, Markham, Ontario, Canada; Hidemia, Mumbai, India; Sigma-Aldrich, St. Quentin Fallavier Cedex, France and Prolabo, Radnor, Pennsylvania, USA, respectively). The pre-culture was prepared by inoculating activated cells into liquid medium YPD (10% yeast extract, 20% fish peptone, 20% glucose) with an initial OD_{600} = 0.25 (UV mini 1240, Shimadzu, Kyoto, Japan) and incubated at 27°C, using a shaker at 150 rpm for 24 h. Cells were harvested using centrifugation (Mirko 220R, Hettich, Tutthingen, Germany) at 2800 x g (5000 rpm) for 5 min at 20°C, re-suspended in sterile distilled water and inoculated into the protein hydrolysate medium at different cells densities. Yeast cultivation was carried out in 250 mL Erlenmeyer flasks incubated at 27°C, using a shaker at 150 rpm for 72 h. The biomass was separated from the liquid using the Mirko centrifuge at 2800 x g for 5 min at 20°C and used to determine the biomass accumulation. The supernatant was collected to be analyzed for crude protein and lipid contents as described in Section 2.5.

2.4 Determination of growth curve, growth rate, biomass accumulation and biomass yield

2.4.1 Determination of growth curves and growth rate

The growth of cells was measured using a cell counting chamber. A growth curve was prepared as a semilog plot of cellular concentration versus time. The growth rate (μ 1/h) was calculated from the growth curve as the maximum slope of the log phase.

2.4.2 Determination of biomass accumulation and biomass yield

Biomass was collected at 72 h of incubation using centrifugation (Mirko 220R) at 2800 x g for 5 min at 20°C and washed three times with distilled water. The biomass was then dried in an oven at 105°C for 3 h and weighed. Biomass accumulation was expressed as dried weight of biomass per

culture volume (g/L). Biomass yield was calculated as dried weight of biomass per lipid used during cultivation (g/g lipid).

2.5 Chemicals analysis

2.5.1 Determination of nitrogen content

Protein content was measured using the Kjeldahl method (TCVN 3705-90, Vietnamese standard) [13] with a conversion factor of 6.25. The nitrogen reduction was calculated as the percentage of nitrogen content in the supernatant versus the initial content in fish protein hydrolysate.

2.5.2 Determination of lipid content

Lipid content was determined using the Bligh-Dyer method [14] with modifications: 4 mL of hydrolysate was weighed and transferred to a glass tube with 7.5 mL chloroform: methanol 1:2 (v/v). The mixture was vortexed (MS2 minishaker, IKA, Wilmington NC, USA) for 1 min, then 2.5 mL chloroform was added and vortexed for another 1 min. The homogenate was centrifuged at 112 x g (1000 rpm) for 5 min at room temperature. The chloroform layer was transferred to a new glass tube using a Pasteur pipette. Another 4 mL chloroform: methanol 1:1 (v/v) was added and centrifuged at 112 x g for 5 min at room temperature. The second extract was mixed with the first and vacuum dried at 35ºC to constant weight (JeioTech OV-01, Lab Companion, Seoul, Korea). Lipid content was calculated as % (g/g). The lipid reduction was calculated as the percentage of lipid content in the supernatant versus the initial content in fish protein hydrolysate.

2.6 Statistical analysis

The different mean values of the measurements were analyzed using a post-hoc test (Tukey's honest significant difference) with a 95% confidence level using Statistica v7.1. The linear relation between the specific growth rate and lipid reduction was evaluated by Pearson correlation coefficient using Microsoft excel. All experiments were conducted 3 times.

3. Results and Discussion

3.1 Growth of *Y. lipolytica* on tuna head protein hydrolysate medium

The head of the tuna is typically the first part removed in common fishery procedures. The yellowfin tuna heads used for hydrolysate preparation contained 19±1% crude protein and 11±1% lipid (results not shown). The yellowfin tuna protein was similar in amount to that found for bigeye tuna and pink salmon, while the lipid content was higher. Bigeye tuna and pink salmon are also considered to be fatty fish [15]. In this study, Protamex, a bacterial protease, was used to prepare the TPH. After removing lipid, the TPH contained 12.5±0.2 g/L crude protein and 0.75±0.01% lipid (Table 1). When compared with common medium YPD that is often used for yeast cultivation, the TPH contents were 2.7-2.8 times higher in protein and 2.5 times higher in lipids, which showed that the TPH was suitable for yeast cultivation.

Table 1. Compositions of media using for yeast cultivation

	Medium	
	Tuna Head Protein Hydrolysates	**YPD**
Protein content (g/L)	12.5±0.2[a]	4.4±0.3[b]
Lipid content (%)	0.75±0.01[a]	0.30±0.01[b]

* Different superscript letters in each row show significant differences at $p < 0.05$. All experiments were conducted 3 times.

The strains *Y. lipolytica* VTCC 0544 was used to explore the ability to grow on TPH. As shown in Table 2, the biomass accumulation was 5.85±0.17 g/L with a specific growth rate of 0.0563±0.0006 (1/h). The strain showed an adequate growth in TPH, compared with YPD which produced 1.3 times more biomass accumulation and 2.2 times higher specific growth rate. Lopes *et al*. [16] reported the same range of biomass accumulation (3.7-8.6 g/L) when *Y. lipolytica* W29 was cultured on medium using pork lard as the sole carbon source. In our study, *Y. lipolytica* VTCC 0544 showed a good ability to use lipids from TPH media as the lipid reduction was 70.3% (Table 2). *Y. lipolytica* was reported to be an oleaginous yeast that could grow on lipids as sole source of carbon [17]. Unlike *S. cerevisiae*, lipid uptake in *Y. lipolytica* was reported to be strongly repressed by glycerol but weakly by glucose [18, 19]. The yeast could assimilate lipids independently even without glucose assimilation. In this study, lipid utilization with glucose medium was 27.4% which showed that lipid uptake occurred whether glucose was present or not. *Y. lipolytica* showed a similar nitrogen utilization with a nitrogen reduction of 19.2-25.4% for both TPH and YPD media. It seems that the protein utilization of *Y. lipolytica* was not dependent on the initial nitrogen content. Chi *et al*. [20] obtained a similar nitrogen reduction (19-35% as calculated from data given in their article) with *Y. lipolytica* ATCC 20460 when grown in municipal wastewater that had a much lower nitrogen content (47.6 mg/L) than the level of TPH (12.5 g/L) in our study. After 72 h of yeast incubation, the average nitrogen content in the TPH was 9.73 g/L, which was 2.1-2.2-fold higher than YPD while its lipid content decreased to 0.21% which was lower than the 0.30% lipid in YPD. This suggests the possibility of producing fish meal or fish peptones as a microbial nitrogen source from the hydrolysates remaining after yeast cultivation.

Table 2. Growth of *Y. lipolytica* VTCC0544 on tuna head protein hydrolysate and YPD

Medium	**Tuna Head Protein Hydrolysates**	**YPD**
Biomass accumulation (g/L)	5.85±0.17[a]	7.69±0.09[b]
Specific growth rate (1/h)	0.0563±0.0006[a]	0.1211±0.0004[b]
Lipid reduction (%)	70.3±0.9[b]	27.4±0.7[a]
Nitrogen reduction (%)	21.5±1.4[a]	26.6±0.6[b]

* Different superscript letters in each row show statistically significant differences at $p < 0.05$. All experiments were conducted 3 times.

3.2 Effect of incubating conditions on growth of *Y. lipolytica* on tuna head protein hydrolysates

3.2.1 Effect of inoculum size

Inoculations were carried out at 5, 10 and 15%. The specific growth rate was slightly decreased from 0.0602±0.0002 (1/h) to 0.0558±0.0002 (1/h) with the inoculum size (Figure 1A). The total nitrogen reduction and lipid reduction were 20.2-22.8% and 70.3-72.6% without significant

differences. The inoculum size did not show a significant effect on biomass accumulation or lipid and nitrogen utilization ($p < 0.05$). The biomass accumulation of 72 h culture was similar for all three inoculations at 6.05±0.18, 5.91±0.05 and 5.98±0.19 g/L, respectively (Figure 1B).

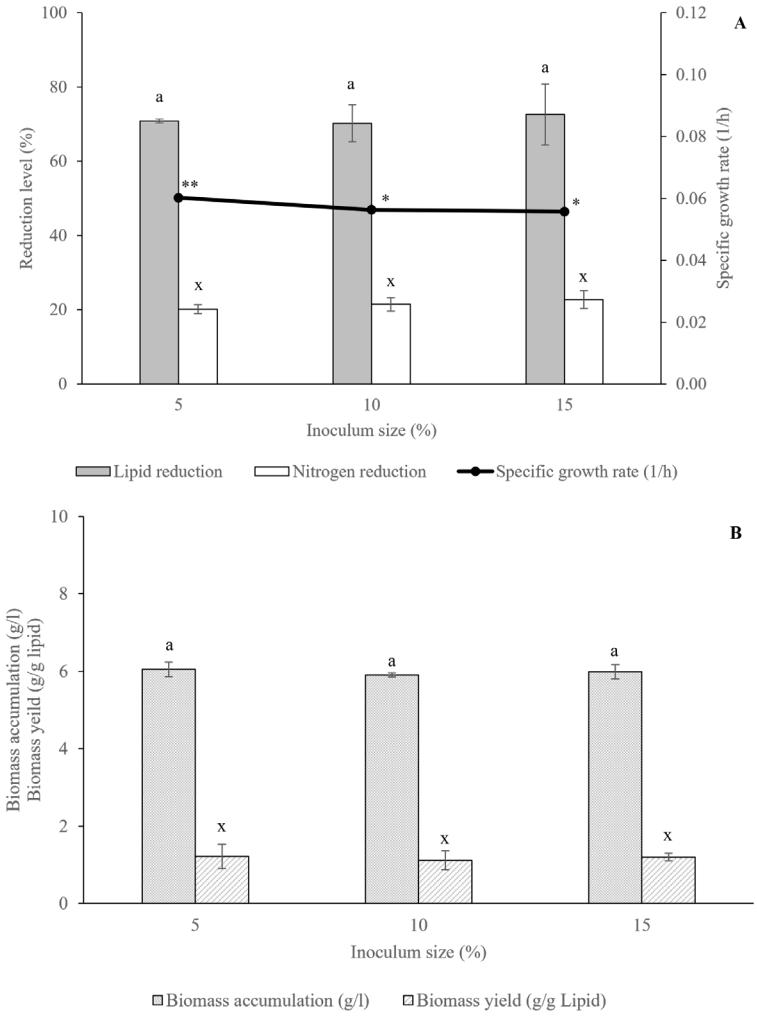

Figure 1. Influence of inoculum size on *Y. lipolytica* growth on tuna head protein hydrolysate

3.2.2 Effect of medium initial pH

Y. lipolytica could grow at a wide range of pH but with different growth rates and biomass production [21]. In this study, the effect of pH on yeast growth was studied over pH range of 5.0 to 7.0. The specific growth rate was increased with pH in the studied range. At pH lower than 6.0, the growth rate was 0.0521-0.0538 (1/h). By increasing pH to 6.5-7.0, the growth rate increased to 0.0563-0.0686 (1/h). A similar tendency of lipid reduction and nitrogen reduction was observed (Figure 2A). The highest lipid reduction and nitrogen reduction were obtained at pHs 5.0 and 7.0, which were 77.6-83.7% and 29.3-38.3%, respectively. The specific growth rate correlated well with

lipid reduction as shown by the Pearson correlation coefficient r=0.77 (p=0.008). The biomass accumulation ranged from 3.73-5.54 g/L. A significantly increased biomass accumulation (5.49-5.54 g/L) and biomass yield (1.09-1.12 g/g) were obtained at pH 6.0-6.5 ($p < 0.05$), a range which included the natural pH of the hydrolysate (Figure 2B). In contrast, a decrease in biomass accumulation was observed at pH 5.0 and pH 7.0. Intracellular pH in *Y. lipolytica* was reported to be 7.0-7.5 [22]. When extracellular pH is different to this value, cells need to spend energy for cytosolic pH maintenance, which can lead to decrement in growth rate [23]. The requirement for energy use can also be compensated by carbon and nitrogen assimilation. A similar result was also reported when studying the growth of the Greek strain *Y. lipolytica* ACA-DC 50109 on stearin [24] which showed that biomass accumulation in *Y. lipolytica* was favored at pH 6.0 but restricted at pH 5.0 and pH 7.0. The respiration activity of *Y. lipolytica* W29 was reported as dependent on pH which was higher at pH 5.6 than at neutral pH (7.0) or acid pH (4.0) [25]. This high rate of respiration may affect the beta-oxidation process which could deviate fatty acids to produce cellular lipid-free materials in yeast cells [26].

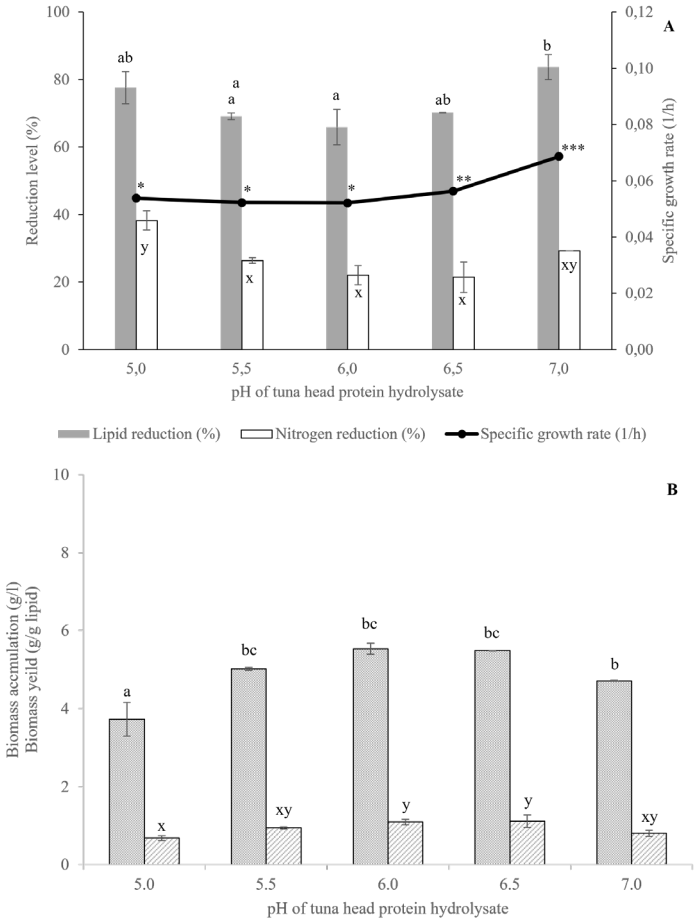

Figure 2. Influence of pH on *Y. lipolytica* growth cultured on tuna head protein hydrolysate

3.2.3 Effect of incubation volume

A series of hydrolysate incubation volumes, 50, 75, 100, 125 and 150 mL was used to cultivate yeast in the shaken flasks. The results show that the specific growth rate decreased with incubation volume. The specific growth rate at 50 mL was 0.0767±0.0008 (1/h), which was 1.6 folds to the one at 150 mL (Figure 3A). In contrast to the specific growth rate, lipid reduction increased with incubation volume. For 50 mL incubation volume, lipid reduction was 60.1±0.7 % while it was 73.8±0.6 % for 150 mL incubation volume. The Pearson correlation efficiency between specific growth rate and lipid reduction was r=-0.82 (p=0.003). Otherwise, there was no significant difference in nitrogen reduction for all investigated incubation volumes. Lipid after entering cytosol can be accumulated in lipid bodies or transported to peroxisome for beta-oxidation [27]. In our work, an increment in lipid content was observed when increasing incubation volume (results not shown) which may explain the higher lipid reduction. When lipids are used as the sole source of carbon by oleaginous yeast, the lipid assimilation is growth-coupled processes [26]. The ex-novo lipid biosynthesis is thereby independent of nitrogen utilization from the culture. This may explain the similarity in nitrogen utilization for all investigated incubation volumes.

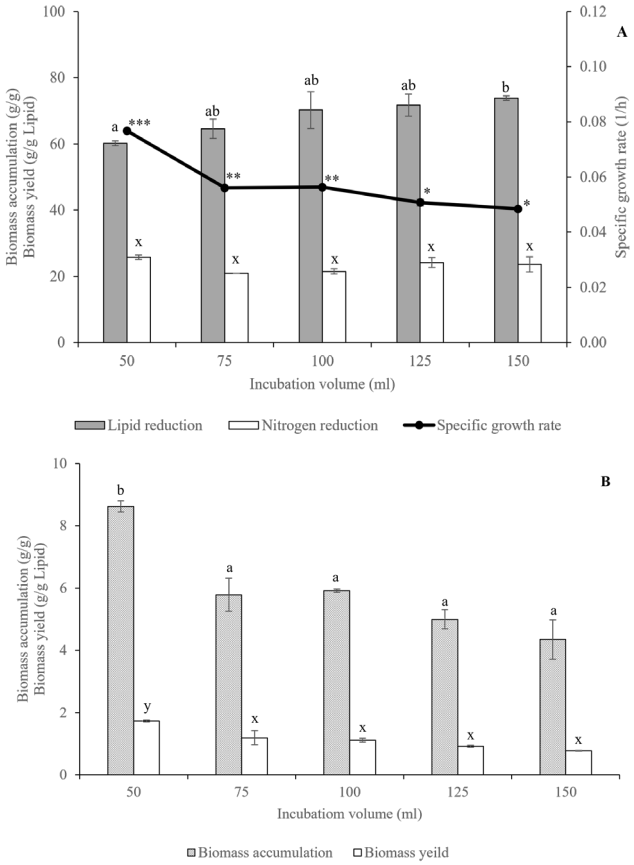

Figure 3. Influence of incubation volume on *Y. lipolytica* growth cultured on tuna head protein hydrolysate

The biomass accumulation was highest at 50 mL incubation volume (8.62±0.19 g/L) and decreased in a volume dependent manner up to 150 mL (4.34±0.63 g/L) (Figure 3B). The culture at 50 mL also produced the highest biomass yield at 1.73±0.03 (g/g lipid). *Y. lipolytica* was known to be a strictly aerobic organism [17]. An increment in incubation volume corresponded to a decrement in oxygenation which inhibited the cell growth. A similar result was reported when a culture of *Y. lipolytica* 583 IMUFRJ 50682 grown on crude oil medium. The results showed that agitation speed had a positive impact on biomass accumulation [28]. Here, in TPH, the main carbon source for yeast growth was lipids. When hydrophobic substrates are used as carbon source to cultivate oleaginous yeast, the substrates are firstly hydrolyzed into free fatty acid which are then transported into the cells. These fatty acids can be used for lipid accumulation in lipid bodies, or can be converted to short chain acyl-CoAs and acetyl-CoAs under activity of acyl-CoA oxidases by beta-oxidation in peroxisome, and are used for energy production providing for cell growth and maintenance [26]. Aerated cultures were reported as having increased acyl-CoA oxidases activities [29] which may have led to increments in biomass production. It was also reported that in highly aerated media, fatty acids were used for fat-free material synthesis in *Y. lipolytica*, which resulted in high biomass production [24]. The results in the present study suggest that oxygen plays an important role in lipid assimilation by the strain.

3.3. Nutritional composition of yeast biomass cultured on tuna head protein hydrolysate

The first parameter used to evaluate nutritional value of yeast biomass is protein content. *Y. lipolytica* cultured on tuna head protein hydrolysate had 64.0±1.3% in protein content, which was higher than previously reported in the literature which ranged from 21-56%, and depended on the strains, cultivation conditions and culture medium (Table 3). Yeast is the most acceptable microorganism for single cell protein production to be consumed. Protein contents in yeast are normally below 60% [30]. *Y. lipolytica* is considered generally recognized as safe (GRAS) and its biomass safety assessment was recently approved by EFSA Panel on Nutrition [31]. The average protein content in *Y. lipolytica* biomass was about 43% and varied depending on the cultured substrates [32]. This content was reported as 30.5-44.5% when the yeast was cultured on glucose-rich hydrolysate obtained from rye and oat agricultural wastes [33]. With glycerol-rich substrates from industrial raw oil waste, the protein content in *Y. lipolytica* biomass was in the range of 45-50.1% [34]. With hydrophobic culture media such as raw rapeseed oil, the protein content of *Y. lipolytica* biomass was reported to be as high as 56.4% [35]. Therefore, there is a promised opportunity to use fatty fish hydrolysate as tuna by-product in microbial protein production.

Table 3. Characteristics of yeast biomass cultured on different media

Yeast Strains	Substrate	Protein Content (%)	Lipid Content (%)	Biomass Accumulation (g/L)	Lipid Productivity (g/L)	Source
Yarrowia lipolytica VTCC0544*	TPH	64.0	9.6	5.92	1.62	This study
Rhodosporidium toruloides Y2	Hydrolysed food wastes	21.7	20.8-25.8	3.2-21.1	0.7-4.3	[10]
Yarrowia lipolytica S6	Crude glycerol	43.0	11.1	12.3	-	[34]
Yarrowia lipolytica ACA-DC 50109	Stearin	-	28	8-9.5	2.7	[24]

* *Y. lipolytica* was cultivated in TPH with natural pH, 100 mL volume incubation.

On the other hand, *Y. lipolytica* is well-known in microbial oil production due to its high capacity for lipid accumulation even when cultured on carbohydrates or lipids as sole carbon sources. In our work, the lipid content reached 9.6±0.7%, which was comparable with the results of Juszczyk *et al*. [34], who cultured *Y. lipolytica* on glycerol. Oleaginous yeasts often produce more than 20% lipids of their dried cell weight. For wild type *Y. lipolytica*, lipid accumulation naturally reached 20-40% [36]. By decreasing oxygenation, the lipid content in *Y. lipolytica* biomass cultured on TPH could reach 33% (results not shown). TPH as a nitrogen rich medium used with hydrophobic carbon source such as fish oil may be a candidate for lipid production using *Y. lipolytica*. In this study, the lipid productivity was 1.62±0.11 g/L, without optimization. This lipid productivity is comparable with the results reported by Dobrowolski *et al*. [37], who cultured *Y. lipolytica* on crude glycerol but lower than reported by Das *et al*. [38], who reported lipid productivity of up to 4 g/L. A much higher lipid productivity of 11.02-16.45 (g/L) was observed when using vegetable oil to cultivate *Y. lipolytica* YB 423-12 [39].

4. Conclusions

Y. lipolytica was successfully cultured on tuna head protein hydrolysates with yeast biomass accumulation of up to 8.62 g/L (dried weight) after 72 h of cultivation, without addition of any external carbon source. The yeast was able to consume up to 83.7% of the lipid in the hydrolysate and retained 70.7% of the nitrogen content. This material could be dried to produce a protein powder. The specific growth rate was in good correlation with lipid utilization. The biomass possessed high protein content, and the work suggested the possibility to be used for lipid accumulation. Further studies should focus on improving biomass and lipid production by addition of supplementary carbon sources, or by controlling the oxygenation of the culture medium. This study demonstrated an approach for environmentally sustainable tuna processing with tuna heads being used to produce three useful products: tuna oil, tuna protein powder and yeast biomass. Moreover, the yeast biomass can be used either as a microbial biomass or as a source of microbial lipids.

5. Acknowledgements

This study was supported by the International Foundation for Science (IFS Grant number E5666-1). We would like to thank Nathalie Persson from IFS for her supervision of the project. Many thanks go to Prof. Joe Regenstein, Cornell University, United States of America, for his enthusiasm and his patience in revising the manuscript for both English and scientific presentation. We acknowledge the support of time and facilities from Nha Trang University and from Ho Chi Minh City University of Technology (HCMUT), VNU-HCM for this study.

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