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REGULAR ARTICLE

COMPARATIVE PRODUCTION OF SINGLE CELL PROTEIN FROM FISH PROTEIN ISOLATE WASTAGE AND ULTRA FILTERED CHEESE WHEY

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ABSTRACT

Fish protein isolate wastage and ultra filtered cheese whey were used as substrates for fermentation by *Kluyveromyces marxianus* to produce single cell protein, under batch and aerobic condition in which pH and temperature were adjusted to 4.5 and 35°C. The produced biomass was analyzed for protein content in different periods of time during fermentation. About 82% and 75% of total protein was produced in the first 18 h of 96 h fermentation of ultra filtered cheese whey and protein isolate wastage respectively, which can be an indication of the exponential phase of the yeast growth. The results of biomass yield measurements during 96 h process also confirm this finding. Moreover, since ultra filtered cheese whey was higher in single cell protein yield, solubility, water holding capacity, water absorption and power of biological and chemical oxygen demand reduction, and also was lower in foam overrun and stability than fish protein isolate wastage, it was selected as the suitable substrate for single cell protein production.

Keywords: Single cell protein, fermentation, biomass, *Kluyveromyces marxianus*

INTRODUCTION

Single cell protein (SCP) production technologies arose as promising ways to solve the problem of worldwide protein shortage. They evolved as bioconversion processes which

turned low-value byproducts, often wastes, into products with added nutritional and market value. Since SCP belongs to one of the cheapest protein products in the market, its production is profitable (**Waites *et al*., 2001**). As compared with plants and animals for providing proteins for food or feed, large-scale industrial production of microbial biomass for the same use has great characteristic advantages such as: Microorganisms in general have a high rate of multiplication and a high protein content (30-80% protein in terms of dry weight); They can utilize a large number of different low cost carbon sources including waste materials (**Lee 1996; Waites *et al*., 2001**).

A special problem with SCP products for human consumption is the nucleic acid content. High content of nucleic acids causes no problems to animals since uric acid is converted to allatonin which is readily excreted in urine. Some practical methods for solving this problem for human use of SCP are alkaline or acidic hydrolysis and activation of endogenous RNA-ases (usually by brief heat treatment at 64°C for 30 min) (**Lee 1996**).

Several processes such as Kiel process in Germany and Vienna process in Austria have been developed for the utilization of lactose in whey to produce SCP. Some of more successful processes were operated by Bel industries in France. The Bel process was developed with the aim of reducing the pollution load of dairy industry waste, while simultaneously producing a marketable protein product (**Waites *et al*., 2001**). While most organisms do not grow on lactose as a carbon source, strains of *Kluyveromyces marxianus* readily grow on lactose (**Ghaly *et al*., 2005**). A number of plants are operated using *Kluyveromyces lactis* or *K. marxianus* (previously named *K. fragilis*) to produce proteins, which are used as a nutritional supplements for both human and animal consumption (**Waites et *al*., 2001**).

On the other hand, increased demands for traditional raw materials to produce fish protein ingredients are leading to great pressure on fish stocks (**Hultin *et al*., 2005**). Due to high level of unsaturated fatty acids, aquatics have a desirable effect on human's health and consequently there is a great interest to consume them (**Pearce and Kinsella, 1978**). Fish protein isolate is a kind of protein ingredient which is prepared from different kinds of raw material, without retaining the original shape of the muscle. Generally, it is not consumed directly and is used as raw material for production of value added products (**Shaviklo, 2006**). The rapidly increasing world population generates the challenge of providing necessary food sources. In particular protein supply poses a problem since essential amino acids can

not be replaced. One possible solution to this problem is SCP production. Bacteria and yeast are candidates for the synthesis of SCP (**Hanson and Hanson, 1981a,b**).

The objective of this paper was to investigate and compare the potential of using fish protein isolate wastage and ultra filtered (UF) cheese whey as substrates under batch fermentation processing to produce SCP by the yeast *Kluyveromyces marxianus*. The information would be useful for the development of a cost effective process in a large industrial scale to produce protein.

MATERIAL AND METHODS

Materials

Ultra filtration permeate cheese whey was kindly provided by Ramak Dairy Factory, 20th km in Booshehr road, Shiraz, Iran. Lyophilized yeast strain *K. marxianus* (PTCC 5193) was obtained from the Persian Type Culture Collection, Tehran, Iran. Ammonium sulphate and hydrochloric acid were from Merck, Germany. All other chemicals were reagent grades and were commercially available.

Whey preparation

UF cheese was drawn from the pipe into 2 L plastic containers. The containers were sealed and transported to the Department of Food Science Laboratory at Shiraz University in Shiraz, where they were stored in a freezer at -20°C until required. Some characteristics of the cheese whey used in this study are presented in Table 1. Prior to fermentation process of cheese whey it was allowed to completely thaw at room temperature for 24 h (**Ghaly and Kamal, 2004; Ghaly *et al*., 2005**).

To reach the highest biomass yield, 0.8 g/L ammonium sulphate as nitrogen source was added to whey. Two liters of raw cheese whey was pasteurized in a bottle (**Ghaly *et al*., 2005; Moeini *et al*., 2004; Waites *et al*., 2001**). The pasteurization technique included heating the whey to 65°C for 30 min, cooling it to 0°C for 30 min and letting it to stand at room temperature (25°C) for 24 h for any spore to germinate. The process of heating, cooling and standing at room

temperature was repeated three times to destroy any vegetative or spore cells present in the whey (**Ghaly and Kamal, 2004; Ghaly *et al*., 2005**).

Fish protein isolate preparation

In order to utilize marine resources and upgrading the by-products of fish filleting, a systematic study was made of the recovery of proteins by chemical extraction (**Batista, 1999**). The proteins of the muscle tissue were first solubilized. The solubilization can be accomplished in 5-10 volumes of water with alkali added to reach approximate pH of 10.5 or higher, or with acid added to get to pH about 3.5 or lower. It is better to choose the pH at which the consistency of the solution decreases to a value that allows the removal of undesirable materials. The mixture was then centrifuged. This allows the light oil fraction to rise to the top of the suspension. At the same time, the lipids of the membrane were removed due to density differences compared to the main protein solution. Other insoluble impurities, such as bone or skin, were also deposited at this stage. The suspension was centrifuged at 6,000 × g for 20 minutes at 4°C for each washing step. The easiest way to precipitate proteins is by adjusting the pH to a value near the isoelectric point of the majority of the proteins that is about 5.2-5.5. Strangely, almost all the muscle proteins become insoluble under these conditions (**Hultin *et al*., 2005; Shaviklo, 2006**). This includes the sarcoplasmic proteins, which are mostly washed away during conventional surimi manufacture. The non-protein soluble materials from the muscle tissue remained in the supernatant fraction after centrifugation and could subsequently be removed. The water remained in the collected protein contained the same concentration of impurities found in the supernatant fraction (**Shaviklo, 2006**). The overall process is illustrated diagrammatically in figure 1 (**Hultin *et al*., 2005**).

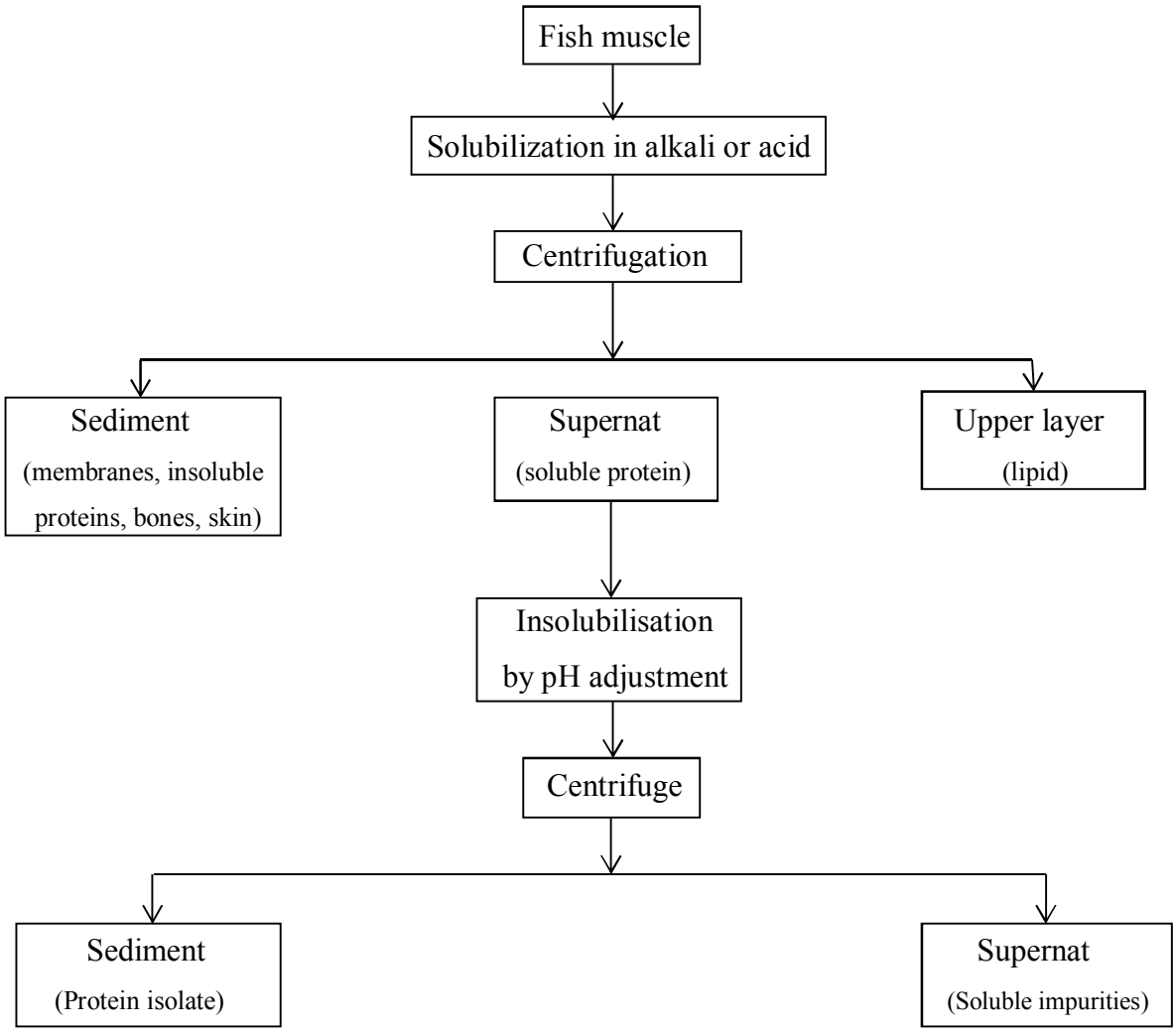

Figure 1 Fish protein isolate process at pHs 3.5 and 10.5, at 6,000 × g for 20 minutes at 4°C for each washing step

Inoculums preparation

Lyophilized yeast strain *K. marxianus* (PTCC 5193) was obtained from the Persian Type Culture Collection, Tehran, Iran. A small amount of a pellet of *K. marxianus* was dissolved in a 7 mL sterilized growth medium tube containing 1% W/V yeast extract, 2% W/V peptone and 2% W/V dextrose. The tube was placed at 30°C for 48 h to activate the yeasts (**Ghaly and Kamal, 2004; Ghaly *et al.*, 2005**).

A loop of this solution was streaked on an agar medium, containing 1% yeast extract, 2% W/V peptone, 2% W/V dextrose and 2% W/V agar in a Petri dish (3

Petri dishes were used). The Petri dishes were then placed in an incubator at 30°C and left until visual growth appeared (after 72 h). Then they stored in refrigerator at 4°C until needed. The yeast colonies were then scooped from the surface of the agar into 80 mL of pasteurized cheese whey and 80 of mL fish protein isolate wastage, in the sterilized Erlenmeyer flasks. The Erlenmeyer flasks were then capped with cotton and mounted on a shaker. The shaker was operated at a speed of 170 rpm for 48 h at 35°C. Using serial dilution method, the number of yeast cells in inoculums was measured to insure the effectiveness of the conditions performed on the shaker (**Ghaly and Kamal, 2004; Ghaly *et al*., 2005**).

Single cell protein production

Two 1 L flasks were filled with 675 mL of pasteurized whey and 675 mL of fish protein isolate wastage. Then, 75 mL of the inoculum (10% by volume) were added to each flask and the contents were mixed thoroughly and distributed in 2 series of 15 flasks (each contained 50 mL). Considering the optimum temperature of *K. marxianus* propagation (30–35°C), the flasks were mounted on a shaker. The shaker was operated at a speed of 200 rpm and temperature of 35°C for 96 h. Next, the flasks were taken out at 0, 6, 12, 18, 24, 72, and 96 intervals in 3 replicates. Immediately after termination of the fermentation process, the flasks were put in water bath at 100°C for 10 min to deactivate the yeasts and interrupt the process. Finally samples were transferred to centrifugation unit to recover the yeast biomass from the spent medium (**Ghaly and Kamal, 2004**).

Biomass efficiency measurement

For measurement of biomass, 40 mL of each sample was transferred to centrifugation tubes in triplicate and centrifuged at 1789 × g for 20 minutes at 4°C. The produced sediment was separated, dried in vacuum oven at 60°C for 8 h and weighed (**Moeini *et al*., 2004**).

Protein measurement

The protein analysis of biomass was performed using AOAC test methods. Protein content was determined by microKjeldahl method with 6.25 conversion factor (**Horwitz, 1985**).

Foaming properties

Foam overrun and stability were measured by whipping the samples with a household type mixer at ambient temperature for 5 min using a graduated glass beaker. Foam overrun was immediately estimated by reading the foam expansion in mL whereas foam stability was measured by weighing the drainage at ambient temperature after 5 min. The referring pHs varied from 2 to 8 by adding solutions of 1 mol/L HCl and 1 mol/L NaOH to each of the sample (**Lieske and Konrad, 1996**).

Emulsification capacity measurement

To measure the emulsion activity and stability, 1 mL of corn oil was added to 3 mL of 1 mg/mL of each the substrates in 0.1 mol/L sodium phosphate, pH 7.4 and the mixture was homogenized at 25 °C for 1 minute. At 1 minute intervals (0 to 10 minutes), 0.1 mL of the emulsion was removed, added to 5 mL of 0.1% SDS solution, absorbance recorded immediately at 500 nm, and plots of absorbance against time prepared. Emulsion activity is the absorbance at zero time and the time required to obtain a 50% reduction in absorbance is a measure of emulsion stability (**Pearce and Kinsella, 1978**). The emulsifying capacity is important from the stand point of the power of SCP to combine with the food ingredients and prevention of SCP loss.

Determination of the amount of water absorbed by single cell protein powders

To measure the amount of water absorbed by single cell protein powders, 1 g of each sample was mixed thoroughly with 10 mL distilled water and put on stirrer for 30 minutes. The samples were remained at room temperature for 10 minutes and the volume of the upper liquid was measured in the 10 ml cylinder (**Batista, 1999**).

Measurement of water holding capacity

To determine the water holding capacity, 2.5 g of the samples was weighed, mixed with 5 ml distilled water, and centrifuged. Water holding capacity is described as the ratio of increase of the sediment's weight to the initial sample's weight (**Van *et al*., 1999**).

Protein solubility at different temperatures

About 0.5 g of dry protein product was accurately weighed, into separate 0.1 L standard beakers and several aliquots of 5.85 g/L NaCl solution were added with stirring to form a smooth paste. Additional 5.85 g/L NaCl solution was then added to bring the total volume of the dispersion to about 0.04 L. Soon after, the mixture was transferred to holding beakers, which circulated hot water inside of them. These holding beakers were coupled to a thermostatic bath, and the temperature was maintained in agreement with the interest of each experiment. In this experiment, the referring temperatures varied from 40 to 90°C by 10°C intervals. After 1 hour the dispersion was transferred to a 0.10 L volumetric balloon, and the volume was completed with NaCl 5.85 g/L. Then the solution was centrifuged to 10188 × g during 30 minutes at 4°C, and the supernatant was then filtered in Whatman paper No. 2. Aliquots of 0.002 L were taken and their soluble protein contents was determined using the micro-Kjeldahl method. The soluble protein percentage was calculated through the following equation (**Morr *et al*., 1985**):

$$P.S. = [A \times 50 / W \times (S/100)] \times 100$$

Where: P.S. = soluble protein content in the sample (g/100g); A = supernatant protein concentration (g/L); W = sample weigh (g); S = sample protein concentration (g/100g).
Each experiment was accomplished in triplicate, being the soluble protein content the resulting average of the three values.

BOD and COD measurement

The BOD test is carried out by diluting the sample with oxygen saturated dilution water, inoculating it with a fixed aliquot of seed, measuring the dissolved oxygen (DO) and then sealing the sample to prevent further oxygen dissolving in. The sample is kept at 20 °C in the dark to prevent photosynthesis (and thereby the addition of oxygen) for five days, and the dissolved oxygen is measured again. The difference between the final DO and initial DO is the BOD.

Chemical oxygen demand (COD) does not differentiate between biologically available and inert organic matter, and it is a measure of the total quantity of oxygen required to oxidize all organic material into carbon dioxide and water. COD values are always greater than BOD values, but COD measurements can be done in a few hours while BOD measurements take five days (**Lenore *et al*., 2003; Sawyer *et al*., 2003**).

RESULTS AND DISCUSSION

Physicochemical properties of Substrates

Table 1 shows some of physicochemical properties of the fish protein isolate wastage and UF cheese whey. The initial pH value of the cheese whey and fish protein isolate wastes used in this study was 7.2 and 7.0, respectively; however the optimum pH for the growth and survival of *K. marxianus* is between 4.0 and 5.0. It has also been recognized that keeping the pH at about 4.5 eliminates possible contamination by lethal bacteria that grow at pH above 6.0. Thus, in this study, pH of the medium was adjusted to 4.5 by the addition of 1 mol/L HCl solution (**Ghaly and Kamal, 2004; Ghaly *et al*., 2005**).

Since the protein content of whey was low, no difficulties related to precipitation of protein during preparation were encountered and therefore UF cheese whey is a more suitable substrate for SCP production than other wheys.

Table 1 Major chemical compositions of fish protein isolate wastage and UF cheese whey

Components	Measured values of fish protein isolate wastage	Measured values of UF cheese whey
Water	99.0 % ± 0.4	93.0 ± 0.3
Ash	0.2 ± % 0.3	0.3 ± 0.7
Protein	0.03 ± % 0.5	0.04 ± 0.1
Lipids	5.8 % ± 0.04	6.1 ± 0.03

Data is expressed as mean ± standard deviation of three replicate samples

Functional properties of the substrates

Single cell protein as a final product had some important functional properties which are shown in table 2. Emulsifying properties of protein samples are ascribed to their amphiphilic properties. After settlement of protein residues at the oil-water interface, the hydrophilic portion aligns to aqueous phase and the hydrophobic part aligns to oil droplets to prevent phase separation. Considering the results, fish protein isolate wastage had better emulsifying properties than UF cheese whey which can be an indication of its protein diversity.

Table 2 Certain functional properties of the substrates

Property	Measured values	
	Fish protein isolate wastage	UF cheese whey
Water absorption	3.8 ± 0.1 g/g sample	4.3 ± 0.3 g/g sample
Water holding capacity	5.1±0.5%	7.5 ± 0.4%
Emulsifying capacity	85.4±0.3%	76.6±0.2%
Emulsifying activity (A_{500})	0.09 ± 0.01	0.06 ± 0.01

Data is expressed as mean ± standard deviation of three replicate samples

Since UF cheese whey had higher water abortion and water holding capacity than fish protein isolate wastage, it is not surprising that UF cheese whey had also higher protein solubility than fish protein isolate wastage at different temperatures. Moreover, increasing of the temperature of the both samples resulted in decreasing of their solubility. Results are shown in the figure 2.

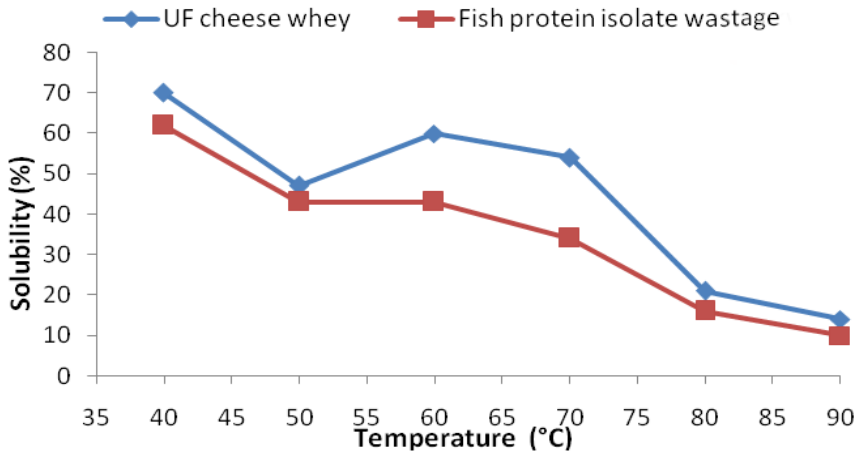

Figure 2 Effect of temperature on the fish protein isolate wastages and whey protein solubility

To be a good foaming agent the protein must be capable of rapid diffusion to the air-water interface and must form a strong cohesive, elastic film by partial unfolding. The foaming properties seem to be correlated with the amount of hydrophobic amino acids that are exposed at the surface of protein molecules (**Lieske and Konrad, 1996**). In the case of fermentation processes like process of single cell protein production, the more foaming capacity of a substrate, the more loosing of the nutritional materials from the surface; therefore, since UF cheese whey has lower foaming capacity than fish protein isolate wastage, it is a better substrate from this stand point (Table 3).

Table 3 Foam overrun of fish protein isolate wastages and UF cheese whey as function of pH

pH	Foam overrun (%)	
	Fish protein isolate wastage	UF cheese whey
2.5	611.1 ± 0.3	515.9 ± 0.1
3.5	615.4 ± 0.1	500.4 ± 0.3
4.5	620.3 ± 0.2	495.8± 0.1
5.5	625.8 ± 0.1	545.2 ± 0.2
6.5	640.4 ± 0.2	525.4 ± 0.1
7.5	650.6 ± 0.1	515.5 ± 0.2
8.5	625.3 ± 0.1	535.4 ± 0.1

Data is expressed as mean ± standard deviation of three replicate samples

To compare the foam stabilities of the substrates with pH, they were plotted against pH. The UF cheese whey and fish protein isolate wastage foams exhibited their best stabilities between pH 4-6 and pH 6-7, respectively ($P < 0.001$). Results are shown in the figure 3.

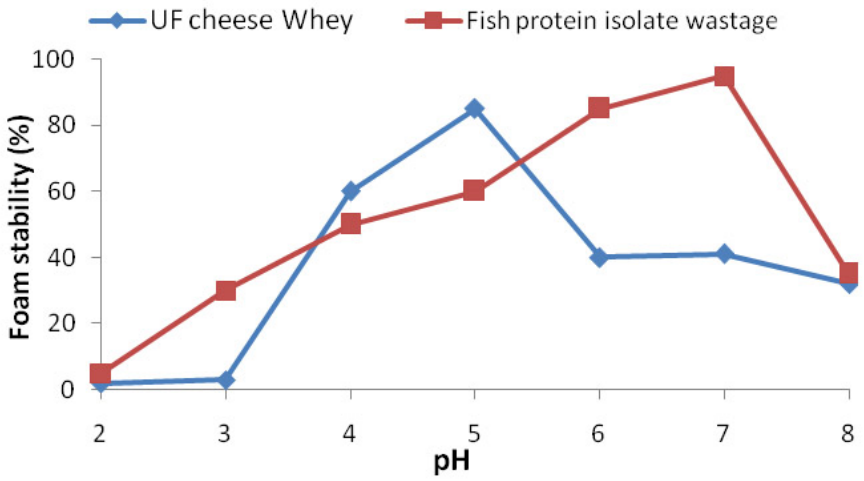

Figure 3 Foam stability of the substrates as function of pH

Because of maximal intra-and intermolecular cohesion, viscosity and hydrophobicity of the substrates, the foam stabilities were higher at or near the each of the substrate's isoelectric point (**Lieske and Konrad, 1996**).

Biomass yield and single cell protein production

Data obtained from the amount of biomass yield corresponds with that of protein produced during fermentation, for each of the two substrates. About 82%

and 75% of total protein was produced in the first 18 h of 96 h fermentation of UF cheese whey and fish protein isolate wastage, respectively. This amount of protein production can be an indication of the exponential phase of the yeast growth (Fig 4 and 5).

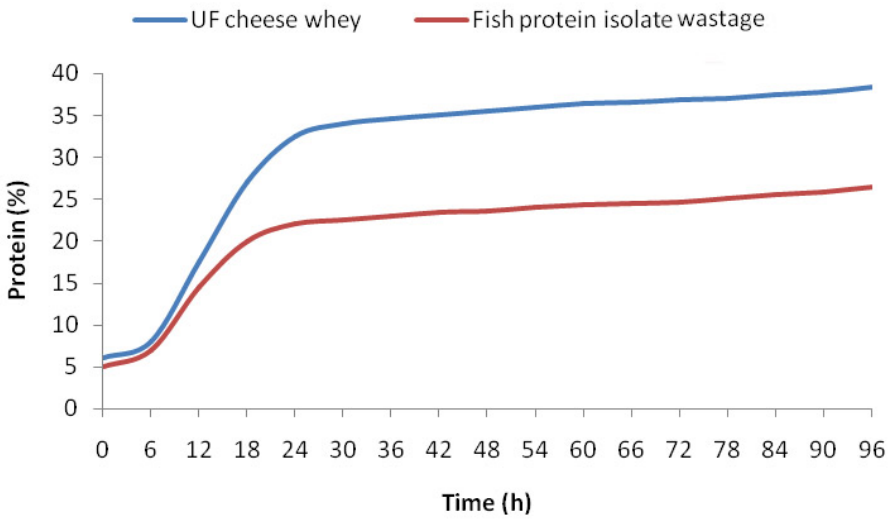

Figure 4 The protein measurement during the batch culturing of *Kluyveromyces marxianus*

Total amounts of protein and biomass yield in 96 h were measured 38.34% and 42.8 g/L for UF cheese whey and 26.4% and 31.2 g/L for fish protein isolate wastage, respectively. These amounts of protein and biomass production are considerable and represent UF cheese whey a better substrate than fish protein isolate wastage under conditions treated (Fig 4 and 5).

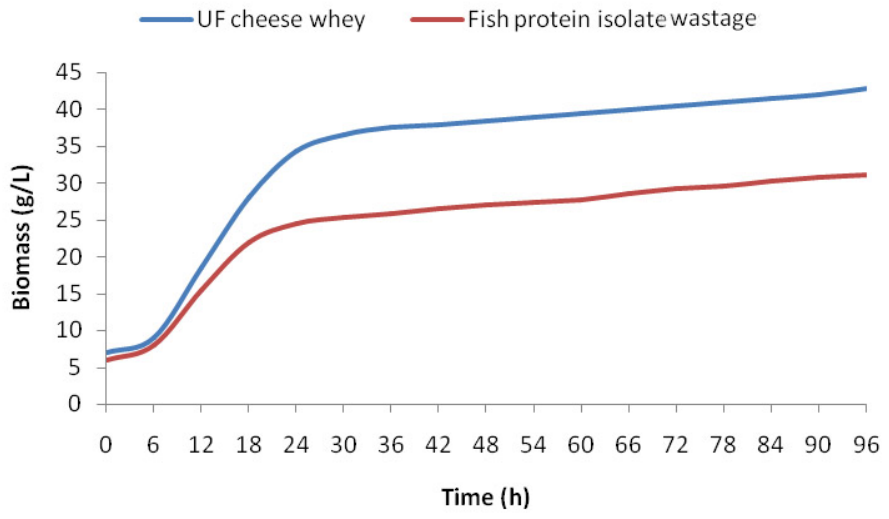

Figure 5 The biomass measurement during the batch culturing of *Kluyveromyces marxianus*

BOD and COD measurement

Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) of UF cheese whey and fish protein isolate wastage were measured before and after fermentation by *K. marxianus* (Table 4). Based on the results, the BOD and the COD of the both substrates were lowered after fermentation, which was an indication of the effects of the yeast cells on them. Moreover, BOD and COD of UF cheese whey were lower than that of fish protein isolate wastage in all cases, which reveals more suitability of this substrate for SCP production than fish protein isolate wastage.

Table 4 BOD and COD measurement

BOD and COD measurement	BOD of UF cheese Whey (ppm)	BOD of fish protein isolate wastage (ppm)	COD of UF cheese Whey (ppm)	COD of fish protein isolate wastage (ppm)
Before fermentation	0.1±25600.2	0.2±29300.3	0.4±51467.4	0.2±57300.3
After fermentation	0.3±14400.1	0.4±17250.5	0.3±38700.2	0.3±41014.

Data is expressed as mean ± standard deviation of three replicate samples

CONCLUSION

With regards to the results obtained from this work, UF cheese whey and fish protein isolate wastage are proper substrates for single cell protein production under the conditions provided in this study, however, for profitable production, in both cases interruption of the fermentation process in the first 18 h, in which the maximum total protein has been produced, can be useful. Moreover, since UF cheese whey was higher in SCP yield, solubility, water holding capacity, water absorption and, power of BOD and COD reduction and also was lower in foam overrun and stability than fish protein isolate wastage, it was selected as the suitable substrate for SCP production. It is recommended to perform further studies to investigate the nucleic acid content and find some ways to reduce it to permitted levels (recommended diary intake is about 2g). Cost effective SCP process can be performed in an

industrial scale and the product can be consumed instead of expensive protein sources present in the market.

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