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Microbial Protein Production from *Candida tropicalis* ATCC13803 in a Submerged Batch Fermentation Process

Sahar Golaghaiee[1], Fatemeh Ardestani[2], Hamid Reza Ghorbani[2]*

1- Department of Chemical Engineering, Shahrood Branch, Islamic Azad University, Shahrood, Iran.

2- Department of Chemical Engineering, Qaemshahr Branch, Islamic Azad University, Qaemshahr, Iran.

Abstract

Background and Objective: Microbial protein production can resolve one of the major world challenges, i.e. lack of protein sources. *Candida tropicalis* growth was investigated to specify a medium to reach the highest cell proliferation and protein production.

Material and Methods: Fractional factorial design and the index of signal to noise ratio were applied for optimization of microbial protein production. Optimization process was conducted based on the experimental results of Taguchi approach designs. Fermentation was performed at 25°C and the agitation speed of 300 rpm for 70 h. Ammonium sulfate, iron sulfate, glycine and glucose concentrations were considered as process variables. Optimization of the culture medium composition was conducted in order to obtain the highest cell biomass concentration and protein content. Experiment design was performed based on the Taguchi approach and L-16 orthogonal arrays using Qualitek-4 software.

Results and Conclusion: Maximum biomass of 8.72 log (CFU ml^{-1}) was obtained using the optimized medium with 0.3, 0.15, 2 and 70 g l^{-1} of ammonium sulfate, iron sulfate, glycine and glucose, respectively. Iron sulfate and ammonium sulfate with 41.76% (w w^{-1}) and 35.27% (w w^{-1}) contributions, respectively, were recognized as the main components for cell growth. Glucose and glycine with 17.12% and 5.86% (w w^{-1}) contributions, respectively, also affected cell production. The highest interaction severity index of +54.16% was observed between glycine and glucose while the least one of +0.43% was recorded for ammonium sulfate and glycine. A deviation of 7% between the highest predicted cell numbers and the experimented count confirms the suitability of the applied statistical method. High protein content of 52.16% (w w^{-1}) as well as low fat and nucleic acids content suggest that *Candida tropicalis* is a suitable case for commercial processes.

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*Corresponding author:
Fatemeh Ardestani
Assistant Professor, Department of Chemical Engineering, Qaemshahr Branch, Islamic Azad University, Qaemshahr, Iran.

Tel: +98-11-42155025
Fax: +98-11-42240091
E-mail:
f.ardestani@qaemiau.ac.ir

1. Introduction

Because of the ever-increasing protein demands worldwide and lack of access to adequate amounts of the common sources of protein, microbial protein has been attracted as a good superseded. Today microbial protein is known as a suitable nutritional fortifier for fish, poultry, pigs and fattening calves in the field of animal feeds as well as soups, baked products and other food products for human consumption.

Now, food source shortage is limited to some Asian, African and South American developing countries. However, it is expected that more advanced countries will face this problem in the future [1]. Protein is one of the major microbial cell components, and the nutritional value of cell biomass is related to its protein content [2]. Microbial protein is produced through bioconversion of agricultural and food industrial residues and wastes into microbial biomass containing high amounts of valuable proteins. Thus, microbial protein production is known as a green process, which is accomplished with wastewater purification via consumption of environmentally pollutant materials [3] such as whey [4], raw glycerol [5], hemicelluloses [6] and cereal processing residues [7] and lignocelluloses wastes [8]. These substrates (carbon and energy sources) could be easily used because of their abundance and cheapness. *Candida (C.) utilis, C. arborea, C. pulcherrima and Saccharomyces* Sp. are common yeasts used for microbial protein production in many cases often for human food and animal feed supply enrichment [9]. However, different other yeasts, microalgae, mold and bacteria have been studied and applied commercially for

microbial protein production. Bacteria have rapid growth, short generation time and high protein content; however, high nucleic acid content is the main disadvantage of bacterial proteins, limiting their public acceptance [10]. Algal protein production is related to warm temperatures, sun light and carbon dioxide, in addition to indigestibility of algal cell wall. Now, modern production methods such as algal cultivation inside photo-bioreactors are developed [11]. Of course, blue-green algae are easily digestible with the most use for lacking cellulose in their cell wall [12]. So, yeasts containing partly high protein content are the most common and favorable microorganisms for protein production [13-15].

Yeasts such as Candida Sp. are well known as non-pathogenic strains for the heterologous production of many industrial valuable products such as enzymes [16], human cytochrome P450s [17], organic acids [18], microbial proteins [19], fermented foods [20] and many other ones.

Microbial protein production is dependent to medium composition and environmental conditions of the process. Type and amount of medium components as well as incubation temperature, pH, dissolved oxygen, moisture content of solid culture medium and some other conditional parameters are known as the most effective items in yeast cell growth and reproduction rate [15]. Microbial protein is produced in both submerged cultures and solid state fermentation. Submerged cultures are preferred for yeasts and bacteria while solid state systems are distinguished to be better for filamentous fungi [21]. In a research project, microbial protein is produced from *Methylococcus capsulatus* inside a U-loop designed reactor to enhance heat and mass transfer and conquer oxygen transport problems [22]. The future of microbial protein production strongly will depend on increasing the fermentation process yield and productivity and at the same time decreasing the prime cost. In this regard, finding high protein content microorganism, improving the process parameters, using cheaper and more accessible waste substrates and also developing low-cost downstream methods could be considered.

Microbial protein production from *C. tropicalis* on acid hydrolyzed rice straw was investigated under different culture conditions including pH, temperature and sugar concentration [23]. Inspite of high protein as well as low fat content of yeasts such as *C. tropicalis*, very few researches have been performed on their applicability for production of microbial proteins. *C. tropicalis* ATCC13803 is a non-pathogenic microbial strain for both humans and animals. However, there is not any new research report on the optimized medium composition for microbial protein production from *C. tropicalis*.

In this work, growth and reproduction of *C. tropicalis* ATCC13803 as suitable non-pathogenic yeast for microbial protein production with high protein and low fat contents was evaluated in a batch submerged culture using different growth factors. A fraction of full factorial methodology was applied to determine an optimal medium composition that led to the highest cell biomass production and protein content yield.

2. Materials and Methods

2.1. Collection of yeast and inoculum preparation

Candida tropicalis ATCC13803 was obtained from American Type Culture Collection (USA) as lyophilized ampoule. Initial medium composition used for primary revival of lyophilized cells contained (g l^{-1}): peptone (Sigma Aldrich, USA), 9; yeast extract (Sigma Aldrich, USA), 10; potassium di-phosphate (Sigma Aldrich, USA), 1; magnesium sulfate (Merck Co., Germany), 1 and glucose (Merck, Germany), 40 [14]. Inoculated cultures were incubated for 24 h at 25°C and 300 rpm agitation speed. Also a few stock cultures of *C. tropicalis* were prepared and maintained on slants of yeast peptone dextrose medium (Yeast extracts 10 g l^{-1}, dextrose (Sigma Aldrich, USA), 20 g l^{-1}, Peptone 20 g l^{-1} and Agar (Sigma Aldrich, USA), 20 g l^{-1}, pH = 6.0) and stored at -20°C. Working cultures were prepared with the same composition on Petri dishes. Cell suspension was provided by collecting the yeast cell colonies grown on the Petri dishes' surface, and transferred to distilled water using a sterile loop under sterile conditions. These suspensions were used as inocula for the next stages (i.e. the main protein production process) [5].

2.2. Medium composition

All media (32 shake flasks) contained some common ingredients including potassium di-phosphate, magnesium sulfate, manganese sulfate (Sigma Aldrich, USA) and zinc sulfate (Sigma Aldrich, USA), each one at a concentration of 1 g l^{-1} in distilled water. To determine an optimized medium composition, four key growth factors (main carbon and nitrogen sources) include ammonium sulfate (Merck, Germany), iron sulfate (Merck, Germany), glycine (Merck, Germany) and glucose concentrations each one at four different levels were considered (Table 1). The experiments were conducted based on the L-16 orthogonal array outcome from Qualitek-4 software (Table 2). Glycine and glucose were prepared and autoclaved separately from all the other medium components to avoid any unwanted destructive reactions. After autoclaving, all medium components were mixed together under sterile conditions, and pH was adjusted to 6.

Table 1. The characteristics of four growth factors selected for optimization of microbial protein production *by C. tropicalis* ATCC13803 in submerged batch culture medium

Serial number	Factor	Level 1	Level 2	Level 3	Level 4
1	Ammonium sulfate Con. (g L^{-1})	0.3	0.4	0.5	0.6
2	Iron sulfate Con. (g L^{-1})	0.02	0.05	0.1	0.15
3	Glycine Con. (g L^{-1})	0.5	1	1.5	2
4	Glucose Con. (g L^{-1})	40	50	60	70

Table 2. The layout of the L-16 orthogonal arrays, produced yeast cell number and calculated S/N for optimization of microbial protein production *by C. tropicalis* ATCC13803 in submerged batch culture medium

Factor	Ammonium sulfate Con. (g L^{-1})	Iron sulfate Con. (g L^{-1})	Glycine Con. (g L^{-1})	Glucose Con. (g L^{-1})	Cell Number*10^{-8} (CFU ml^{-1})		S/N
Trial		Factor level			Repeat 1	Repeat 2	
1	1	1	1	1	2.91±0.001	3.22±0.002	9.69
2	1	2	2	2	3.31±0.002	3.54±0.002	10.68
3	1	3	3	3	0.74±0.001	0.93±0.001	-1.74
4	1	4	4	4	4.56±0.001	4.82±0.002	13.41
5	2	1	2	3	1.86±0.003	2.02±0.001	5.73
6	2	2	1	4	1.65±0.001	1.91±0.003	4.94
7	2	3	4	1	1.03±0.003	1.41±0.001	1.41
8	2	4	3	2	1.82±0.001	2.20±0.001	5.95
9	3	1	3	4	3.30±0.001	3.53±0.001	10.65
10	3	2	4	3	1.23±0.001	1.43±0.001	2.40
11	3	3	1	2	0.29±0.002	0.72±0.002	-8.39
12	3	4	2	1	1.68±0.002	1.97±0.002	5.14
13	4	1	4	2	1.16±0.003	1.51±0.001	2.28
14	4	2	3	1	0.96±0.001	1.11±0.001	0.24
15	4	3	2	4	0.49±0.001	0.76±0.002	-4.69
16	4	4	1	3	3.07±0.003	3.54±0.001	10.32

2.3. Fermentation process for microbial protein production

Microbial protein production was done in 250 ml Erlenmeyer flasks containing 100 ml sterile medium with different compositions as designed by the software (Table 2) in a rotary shaker incubator (Fan Azma Gostar, KM65, Iran) for 50 h. All flasks were inoculated using 1 ml of *C. tropicalis* ATCC13803 cell suspension (~1×10^8 CFU ml^{-1}), and then incubated at 25°C with an agitation rate of 300 rpm for 70 h. Each experiment was repeated for two times, and the mean values were considered as final data.

2.4. Measurements

After 70 h incubation, the shake flasks contents were used as the analytical samples. At this time, the medium seemed opaque from yeast cell growth and reproduction. Cell number was assayed using a spectrophotometer (JENWAY, 6310, UK) at a wavelength of 600 nm. Ten standard solutions of *C. tropicalis* ATCC13803 with assigned cell number were prepared and used to determine the related standard curve. An un-inoculated sample of medium without any yeast cell was used as control.

Each shake flask's content was filtered by using a micro-filter with a pore size of 0.2 µm and then centrifuged at 2000 ×g for 10 min. The supernatants were used for glucose concentration measurement. The glucose concentration was measured by a colorimetric method using the dinitrosalicylic acid (DNS) reagent (Merck, Germany) and a spectrophotometer (JENWAY, 6310, UK) at a wavelength of 540 nm [24].

Total nitrogen was determined by micro-Kjeldahl method according to Iran National Standard Organization method (INSO 19052). Digestive catalyst contained K_2SO_4, $CuSO_4.5H_2O$ and TiO_2 (Merck Co., Germany) [25].

Nucleic acids content of the cell biomass was determined using spectrophotometer (JENWAY, 6310, UK). Nucleic acid separation was conducted using acid guanidinium thiocyanate-phenol-chloroform mixture [26]. Total fat of the cell biomass was measured using gravimetric method [27].

3. Results and Discussion

3.1. Cell growth and reproduction

Number of produced yeast cells (presented in Table 2) shows that maximum yeast cell number (8.66 log CFU ml^{-1} in the first trial and 8.68 log CFU ml^{-1} in the second trial) was obtained in the experiment number 4; the concentration of ammonium sulfate, iron sulfate, glycine and glucose was adjusted on 0.3, 0.15, 2 and 70 g l^{-1}, respectively (Table 2). Minimum yeast cell number (7.46 log CFU ml^{-1} for the first trial and 7.86 log CFU ml^{-1} for the second trial) was obtained in the experiment number 11; the concentration of ammonium sulfate, iron sulfate, glycine

and glucose adjusted on 0.5, 0.1, 0.5 and 50 g l^{-1}, respectively (Table 2).

3.2. Effect of each factor's level on cell growth and reproduction

Signal to noise ratio (S/N) was calculated for each trial (Table 2) based on Eq.1:

$$\frac{S}{N} \equiv -10Log\left(\frac{\sum_{i=1}^{n}(1/y_i)^2}{n}\right) \quad \text{Eq. 1}$$

Where, n is the number of experiment repeating, and y_i is the number of yeast cells in each treatment.

The average effect of each factor at the designed levels on cell reproduction is presented in Figure 1.

The results showed that ammonium sulfate at level 1 (0.3 g l^{-1}), iron sulfate at level 4 (0.15 g l^{-1}), glycine at level 4 (2 g l^{-1}) and glucose at level 4 (70 g l^{-1}) had the most effectiveness on the growth and proliferation rate of *C. tropicalis*. Increasing the concentration of ammonium sulfate caused to a decrease in the produced cell numbers. While other factors showed different trends as higher cell proliferation at the greater levels of the nutritional agent.

3.3. Interaction between factors

The investigated cases for the mentioned two factors interaction are presented separately in Table 1. The highest interaction between glycine and glucose concentrations and the least interaction between ammonium sulfate and glycine concentrations were obtained (Table 3). The interaction severity index for both of the factors was calculated by Qualitek-4 software (Table 3). Figure 2 represents S/N values in different experiments for the two studied factors based on their interaction with each other, and confirms the results expressed in Table 3. It is to be noted that Figure 2 has been drawn by Qualitek-4 software based on the combination of the calculated S/N ratios for each experiment. For example, Figure 2 (a) demonstrates the interaction between glucose and glycine. Each point represents the amount of S/N ratio for one of the conducted experiments. The mentioned experiment is addressed via the applied level of glucose (horizontal axis) and glycine (the legend).

The highest interaction was observed between glycine and glucose concentrations with interaction severity index equal to +54.16% (Table 3). Also the least interaction was recorded between ammonium sulfate and glycine concentrations with interaction severity index equal to +0.43%. The results indicated that interaction between iron sulfate and glycine concentrations, as well as the interaction between iron sulfate and glucose is considerable too. The higher interaction severity index indicates more interference effects resulted from the influence of each factor in the cell culture medium on the other one. This means that, the presence of glycine in *C. tropicalis* ATCC13803 culturing medium can potentially affect glucose functionality by as much as +54.16% and vice versa. Also it can be deduced that ammonium sulfate and glycine do not have any significant interference impact on each other.

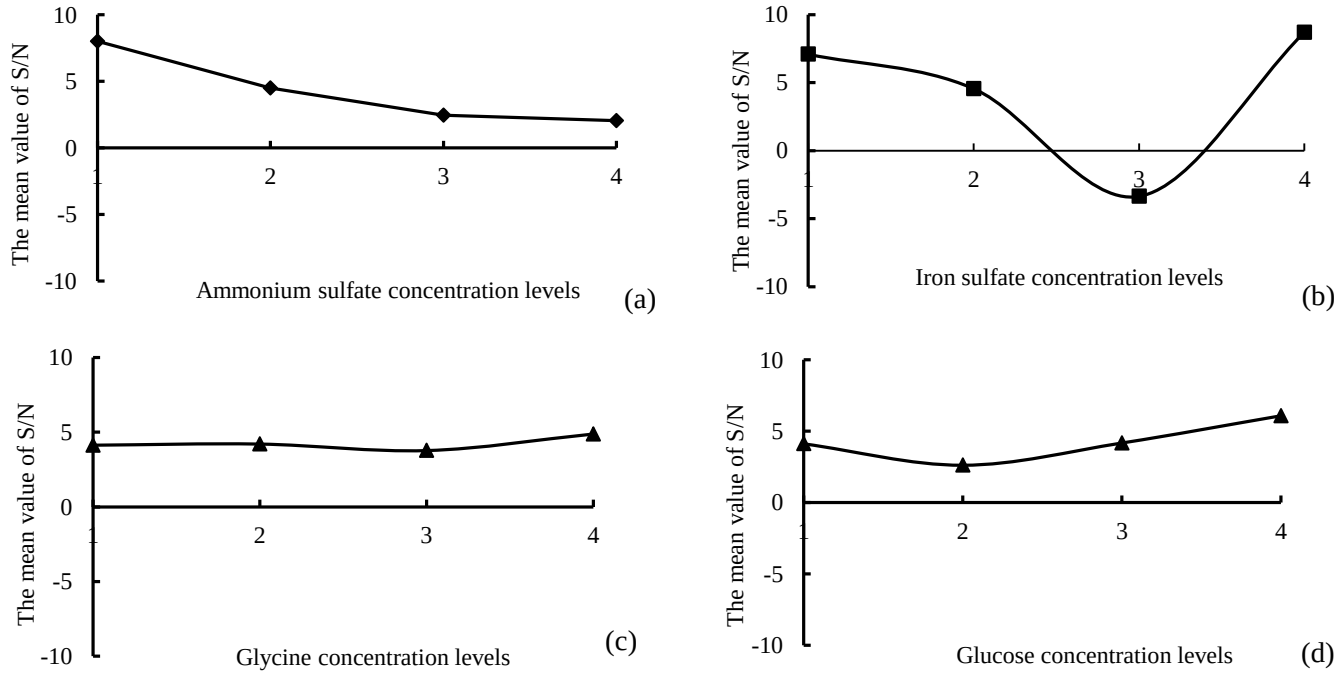

Fig 1. The main effect of each factor on the cell growth in optimization of microbial protein production *by C. tropicalis* ATCC13803 in submerged batch culture medium

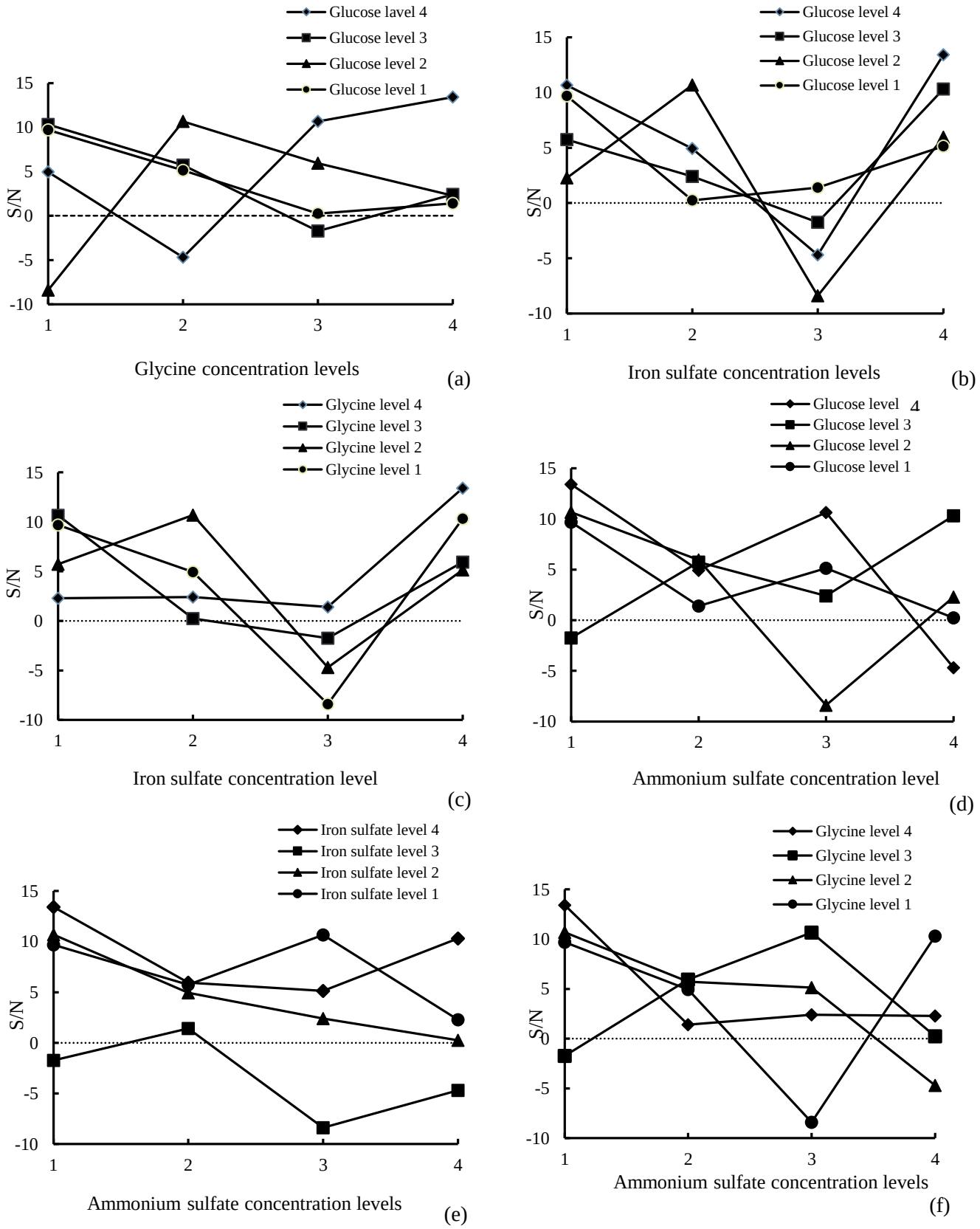

Fig 2. Factor interactions for optimization of microbial protein production *by C. tropicalis* ATCC13803 in a submerged batch culture medium (a): Glycine and glucose, (b): Iron sulfate and glucose, (c): Iron sulfate and glycine, (d): Ammonium sulfate and glucose, (e): Ammonium sulfate and iron sulfate, and (f): Ammonium sulfate and glycine

Table 3. Interaction between factors in optimization of microbial protein production *by C. tropicalis* ATCC13803 in submerged batch culture medium.

Factors	Interaction Severity Index (%)
Glycine * Glucose	54.16
Iron sulfate * Glucose	40.90
Iron sulfate * Glycine	22.24
Ammonium sulfate * Glucose	8.14
Ammonium sulfate * Iron sulfate	4.07
Ammonium sulfate* Glycine	0.43

3.4. Optimized medium composition

Optimized condition was determined with ammonium sulfate, iron sulfate, glycine and glucose concentrations equal to 0.3, 0.15, 2 and 70 g l^{-1}, respectively. Under these conditions, using the proposed medium composition, it is expected to reach to 8.75 log CFU ml^{-1} theoretically. However, the real obtained value was equal to 8.72 log CFU ml^{-1} with only 7% standard deviation. The highest difference between the produced cell numbers using levels 1 and 2 of a factor was attributed to ammonium sulfate concentration. The appointed difference was equal to -3.51 units of S/N value. A minor difference of 7% between the theoretical and experimental data on produced cell population under proposed optimized conditions confirms the susceptibility of fraction of full factorial methodology to determine the optimal medium composition. Iron sulfate and ammonium sulfate concentrations were recognized as the most effective factors on *C. tropicalis* growth and cell production with 41.76% and 35.27% (w w^{-1}) allotment, respectively. Thus glucose and glycine concentrations had inconspicuous roles in *C. tropicalis* growth with 17.12% and 5.86% (w w^{-1}) contribution, respectively. Dry cell biomass concentration of 0.49 g l^{-1} for *C. tropicalis* on the 7^{th} day of fermentation at 5% concentration of pineapple waste (0.52 g l^{-1} obtained dry cell biomass at optimized conditions) has been recorded by a previous research with a good compliance to our results [28]. *Bacillus (B.) subtilis* NCIM 2010 growth on whey substrate in a submerged fermentation has been investigated and the results showed a maximum cell biomass concentration of 0.32 g l^{-1}, which is slightly more than half of the present work results on *C. tropicalis* ATCC13803 [4].

3.5. Evaluation of microbial protein production

Maximum extracted protein in the dried cell biomass of *C. tropicalis* ATCC13803 using optimized medium composition was measured to be equal to 52.16% (w w^{-1}). The obtained protein content in the biomass is very considerable in compare to that obtained in many previous researches for other microbial protein producer strains. Maximum protein content of 35% in dry cell biomass for *Aspergillus terreus* using an optimized medium composition has been reported by a previous research that is about 33% lower than our obtained result [29]. Also a protein content of *C. tropicalis* on the 3^{rd} day of the incubation using 5% concentration of pineapple waste has been recorded as about 48% that is a little less, and however, in agreement with our results [28]. The obtained protein of *C. tropicalis* ATCC13803 is significantly more than 30.4% protein content of *Aspergillus niger* AS-101[30]. A local Philippine isolate *C. tropicalis* on acid hydrolyzed rice straw at pH 5.5, 29°C and 1.6% sugar concentration resulted to 51% protein in the cell biomass that is near to our findings [23].

Saccharomyces cerevisiae biomass containing 49.29% crude protein has been obtained by a previous research, which is less than our finding in the present work [31]. For *Saccharomyces cerevisiae* cell biomass, banana skin was distinguished as the best substrate in compare to apple and mango wastes as well as sweet orange peel with 58.62% crude protein; this is slightly more than our results for *C. tropicalis* [32]. A maximum protein obtained from *Apergillus (A.) oryzae* equal to 57.3 mg per 100 g of a mixture of pomegranate rind and guava peel as the main substrate and also from *Rhizopus (R). oligosporus* equal to 61.2 mg per 100 g of a combination of pineapple skin and pomegranate rind as the main substrate has been reported [33]; whereas only about 20% (w w^{-1}) protein content for *Yarrowia lipolytica* NRRL YB-423 on glycerol as a raw carbon source material has been reported [5]. The produced protein by *Kluyveromyces (K.) marxianus* CBS 6556 on sweet and sour whey proteins showed considerable higher levels of valine, leucine, isoleucine, threonine, phenylalanine, tyrosine and some other essential amino acids in compare to sweet and sour whey proteins [34].

The percentage of fat and nucleic acids in the obtained dry biomass of *C. tropicalis* ATCC13803 was obtained as 2.5% and 8.3% (w w^{-1}), respectively. Low fat content and low nucleic acid level are among the most important advantages of *C. tropicalis* ATCC13803, making it suitable for commercial microbial protein production in compare to bacterial strains with high nucleic acid levels. Since, high levels of nucleic acid (more than 2 g per day) in human diet could cause to some health disorders such as formation of kidney or gout stone, the maximum permissible adding amounts of microbial proteins in human food or animal feeds has been limited [1].

4. Conclusion

This is the first report on *C. tropicalis* ATCC13803 growth and reproduction with emphasis on optimization of the medium composition using the experimental results of the fractional factorial design. Produced cell biomass and protein content at optimized conditions were considerable in compare to some other protein producer microbial species. The changes in iron sulfate and ammonium sulfate concentrations showed significant effects on *C. tropicalis* growth. *C. tropicalis* is sensitive to high levels of

ammonium sulfate concentration. Due to high protein content as well as low levels of fat and nucleic acids obtained in the dry cell biomass of *C. tropicalis* ATCC13803, there is a good potential to commercialize this process.

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6. Conflict of interest

The authors declare that there is no conflict of interest.

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