Design of Experiment Methodology for Mannoprotein Industrial Scale Manufacturing Bioprocess: Optimization from a Yeast Cell Factory

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ABSTRACT: *Kluyveromyces marxianus is a yeast species with various industrial applications. It's known for its ability to metabolize a wide range of substrates, including lactose, xylose, and cellobiose, which makes it useful in various biotechnological processes. In order to Design of Experiments (DOE) and design an optimized method for extracting and purification of mannoprotein using three homogenization, alkalinity, and bio-emulsifier extraction methods, as well as model data at three levels based on three factors: acidity, temperature, and the mannoprotein extraction method, this study looked at the growth of the yeast Kluyveromyces marxianus (IBRC-M 30114) in a 30-L fermentor to determine its optimized yield. Mannoprotein was extracted (in 27 runs with 3 parts) using QUALITEK-4 software and the Taguchi method. In the designed stage, various temperatures (25, 30, and 35 ℃) and pHs (2, 5, and 7) were used in the homogenization, alkaline, and bio-emulsifier methods. The growth of the yeast K. marxianus in a fermenter (bioreactor) showed that the maximum biomass was obtained from the scale increase inside the reactor. This indicated that the maximum fermentation biomass 34.02 (g/L) was obtained from K. marxianus in 30 h at pH 4.6, 29 ℃, 500 rpm, oxygen 19.7, inlet air volume 1 vvm[3], and 36.6 DO. The maximum amount of mannoprotein 8.243 (mg 100 m/L) from 10 (g/L) yeast biomass was extracted by the alkaline method with pH 5. In the bio-emulsifier method, the mannoprotein extraction was maximized at pH 7. The homogenization approach fared better than the alkaline method overall in terms of performance. In contrast, the alkaline method outperformed the other two methods homogenization and bioemulsifier in terms of volume or quantity. Mannoproteins play important roles in various biological processes and have several applications in different industry. Mannoproteins have potential applications in drug delivery and as carriers for bioactive compounds due to their biocompatibility and ability to interact with cells and tissues.*

***KEYWORDS*:** *Mannoprotein, K. marxianus, Optimization, Design of Experiment (DOE), Bioprocess*

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INTRODUCTION

Kluyveromyces marxianus, often referred to as *K. marxianus*, is a thermotolerant yeast with multiple biotechnological potentials for industrial applications, which can metabolize a broad range of carbon sources, including less conventional sugars like lactose, xylose, arabinose, cellobiose and inulin. These phenotypic traits are sustained even up to 45 °C, what makes it a relevant candidate for industrial biotechnology applications. It is therefore of much interest to get more insight into the metabolism of this yeast [1-10].

The use of *K. marxianus* for industrial-scale mannoprotein production is relatively unexplored compared to other microorganisms like SamLharomyces cerevisiae. Investigating its potential in this context is a novel approach. On the other hand SamLharomyces cerevisiae and *Kluyveromyces marxianus* are both yeast species used in various industrial applications, but they have distinct advantages depending on the specific industry and application [11-13].

Some of *K. marxianus* industrial applications include: ethanol production, baking, bioconversion of lignocellulosic biomass, food additives and flavors and its ability to metabolize a wide range of substrates [14,15].

K. marxianus can also utilize xylose, a sugar present in lignocellulosic biomass, which is challenging for many other yeasts. This makes it valuable for bioconversion of agricultural residues into biofuels and other products [16].

Acording to growth conditions, *K. marxianus* prefers aerobic (oxygen-rich) conditions and can grow over a wide pH range (typically between 4 and 8). It can tolerate higher temperatures compared to other yeasts, with an optimal growth temperature ranging from 37 to 42°C. Also, its growth on different substrates can vary, and therefore it's important to optimize its growth conditions for specific applications [17,18].

Here are some advantages of *K. marxianus* over SamLharomyces cerevisiae in certain industrial approaches. *K. marxianus* can thrive at higher temperatures compared to SamLharomyces cerevisiae. This thermotolerance is advantageous in industries like bioethanol production, where higher fermentation temperatures can be used to increase productivity [1,6]. *K. marxianus* can ferment a wider range of substrates, including lactose and xylose, which are not efficiently metabolized by SamLharomyces cerevisiae. This makes it valuable in processes involving dairy waste or lignocellulosic materials. *K. marxianus* is known for its high mannoprotein production, which has applications in the food and beverage industry for improving the mouthfeel and stability of products [1,17,18].

The cell wall protects the *K. marxianus* yeast from stress and has a complex molecular structure, composed of an external part of mannoproteins. Mannoproteins are very interesting molecules, owing to their functional properties, dependent on their molecular features with massive mannosylations. Therefore, the molecular characterization of mannoproteins is a must, although the results relying on the optimal isolation and preparation of this cell wall fraction [19].

Mannoprotein from Yeast Cell Wall is a large family of natural compounds in which polysamLharides are connected to proteins and peptides by covalent and non-covalent bonds. The structures and molecular weights of mannoproteins vary, depending on the degree and type of glycosylation. The polysamLharide chains consist almost exclusively of mannose units linked together by α-links, with a long α-1→6 linked backbone containing short α-1→2- and α-1→3 linked side chains. Several of the side chains may have phosphodiester linkages to other mannosyl residues. Mannoproteins have molecular weights ranging from below 20 kDa to more than 450 kDa [20,21].

Mannoproteins play important roles in various biological processes and have several applications in different industries. Mannoproteins are a major component of the cell wall in yeasts and fungi. They contribute to the structural integrity of the cell wall and help maintain cell shape. Some key points about mannoproteins and their applications are: cell wall structure, flomLulation and sedimentation, stabilization and mouthfeel in oral products, food and beverage industry, yeast nutrition and health, cosmetics and personal care, diagnostic applications [22].

Mannoproteins are versatile molecules with a range of applications in various industries. Their unique properties, including their interactions with other molecules and their impact on cellular behavior, make them valuable components in diverse fields of science and technology [23].

The cell wall of the yeast *K. marxianus* is a valuable source for extracting mannoprotein with high efficiency. The design, optimization of the production, and extraction processes of mannoprotein from *K. marxianus* were examined to use it [24]. Mannoprotein production by this yeast species through combining the fractional factorial

method with optimization by the response level was reported in a study in 2020. It was found that the concentration of the carbon and nitrogen sources, as well as fermentation time and pH, had the most effects on mannoprotein production [25].

Mannoprotein was originally extracted from the cell wall of *S. cerevisiae* by *Cameron et al*., and later, *Moreira et al*. described its effect as a biopolymer-forming liquid [26]. *Dicket et al*. extracted purified mannoprotein from *S. cerevisiae* KA01 by autoclaving in a buffer and dialyzing in distilled water, respectively [27]. *Wan et al.* examined different methods to extract mannoprotein from the cell wall of *S. cerevisiae*. They observed that extraction in warm water of hexadecyltrimethylammonium bromide [$C_{16}H_{33}N(CH_3)_3Br$] was the optimized method with the highest protective effects on the structure of mannoprotein [28].

Prima et al. tried to produce glutathione from mannan-based biological sources using *S. cerevisiae*. They reported mannoprotein as a valuable source of carbon for producing glutathione. Mannoprotein hydrolysis in *S. cerevisiae* was also developed by the inhomogeneous expression of mannanase/mannosidase on its cell surface [29].

Mannoprotein was first extracted from the cell wall of *SamLharomyces cerevisiae* and introduced as a bio-emulsifier with high extraction efficiency compared to other microbial resources [30-32]. Compounds with emulsifiers' types and structures can be used as adjuvants in AS03 (Influenza-pandemic) vamLines and facilitate the process of local production of cytokines and absorption of intrinsic cells. Previous studies manifest that compounds with emulsifier types and structures can be used as adjuvants in AS03 vamLines and may facilitate the local production of cytokines and the uptake of intrinsic cells [33].

The alkali method is a commonly used technique for the extraction of mannoproteins from yeast cells. It involves treating yeast cells with an alkaline solution to disrupt the cell walls and release the mannoproteins [34-38].

Homogenization is a mechanical method used for the extraction of mannoproteins from yeast cells. It involves breaking down the cells using physical force to release the intracellular components, including mannoproteins. Homogenization can efficiently disrupt yeast cells, leading to the release of intracellular components, including mannoproteins. The method might yield a mixture of various cellular components, so further purification steps could be necessary for obtaining highly pure mannoproteins. The choice of homogenization method and buffer can influence the yield and quality of extracted mannoproteins. Optimization of homogenization parameters (pressure, number of passes, etc.) is crucial to achieving effective cell disruption without denaturing the proteins [39-44].

The bioemulsifier method is not a common approach for the extraction of mannoproteins from yeast cells. Bioemulsifiers are biomolecules produced by microorganisms that have emulsifying properties, often used to stabilize oil-in-water emulsions. Extracting mannoproteins using a bioemulsifier method might involve utilizing the emulsifying properties of these molecules to facilitate the release of mannoproteins from yeast cells. However, this approach might not be as established or widely used as other extraction methods [45-48]

Designing experiments (DOE) for the production and purification of mannoproteins from yeasts involves systematically varying factors to optimize the process. Factors to consider: carbon source (glucose, sucrose, lactose); nitrogen source (ammonium sulfate, yeast extract); induction time; pH and temperature; cell density; cell disruption method; elution conditions; concentration and buffer exchange [49].

A variety of software is used in designing experiments, including Statease, MiniTab, Qualtek, etc. Although Minitab software is more common for designing experiments, it has limitations in some parts of the Taguchi method, including the simultaneous examination of several answers with different features. Thus, Qualitek-4 software is usually recommended as it is specially designed for the Taguchi method. These tables are selected for different test situations, among the total number of experiments in the complete factorial method, and help to examine several factors at different levels simultaneously based on their interactions. The Taguchi method identifies experiments with the greatest effects of selected factors among all possible scenarios [50-52].

The novelty of this research topic "Design of Experiment for Mannoprotein Industrial-Scale Manufacturing Bio Process Optimization from *K. marxianus*" lies in several aspects:

Production point of view, DOE point of view, Scale of production point of view and Bioprocess engineering point of view. Using a appropriate method for mannoprotein extraction might require a good understanding of the properties and its compatibility with yeast cells and mannoproteins.

Table 1: Materials for mannoprotein extraction methods

Alkaline	Bio-emulsifier	Homogenization
Yeast cells containing mannoproteins	Yeast cells containing mannoproteins	Yeast cells containing mannoproteins
Alkaline solution (e.g., sodium hydroxide, NaOH)	Bioemulsifier-producing microorganisms or commercial bioemulsifier	Homogenization buffer (physiological buffer)
Buffer solution for neutralization	Buffer solutions	Homogenizer (mechanical disruptor)
Centrifuge or filtration equipment		Centrifuge or filtration equipment

Bio-emulsifier method might not be as well-established as other extraction techniques (homogenization and alkali methods), so careful optimization and testing are important to ensure effective and reliable results. Therefore, the extraction of mannoprotein from the yeast *K. marxianus* is of special importance. This study aimed to optimize mannoprotein extraction from *K. marxianus* (IBRC-M 30114) to the highest level and to design an optimized method for mannoprotein extraction.

EXPERIMENTAL SECTION

Materials

Materials for preparing a fermenter (bioreactor) for mixing liquid

The biotransformation process was performed in a 30-L sterilized and filtered fermenter (bioreactor), including 300 ml of an optimized implantation environment and a formerly planted Erlenmeyer. To achieve the maximum growth rate of the yeast, the fermenter included 55.15 g/L of glucose, 9.35 g/L of the yeast extract with pH 4.99, and a fermentation time of 168 h (16). Each liter of the fermentation implantation environment included 40 mL of glycerol, 5.0 mL of H_2SO_4, basal salt medium (26.7 mL of 85% H_3PO_4, 0.93 g of $CaSO_4$, 18.2 g of K_2SO_4, 14.9 g of $MgSO_4 \cdot 7H_2O$, 4.13 g of KOH), trace mineral mix (6.0 g of $CuSO_4 \cdot 5H_2O$, 0.08 g of NaI, 3.0 g of $MnSO_4 \cdot H_2O$, 0.2 g of $Na_2MoO_4 \cdot 2H_2O$, 0.02 g of H_3BO_3, 0.5 g of $CoCl_2$, 20.0 g of $ZnCl_2$, 65.0 g of $FeSO_4 \cdot 7H_2O$) and a vitamin mix (128 mg of $C_6H_{12}O_6$, 12.8 mg of $C_{18}H_{32}CaN_2O_{10}$, 12.8 mg of $C_8H_9NO_3 \cdot HCl$, 64 mg of K_2HPO_4, 3.2 mg of thiamine hydrochloride (vitamin B1), 0.2 g of nicotinic acid (vitamin B3), and 12.8 mg of D-Biotin). All the materials and implantation environments were obtained from the Merck Company (Germany).

Materials for mannoprotein extraction

K. marxianus (IBRC-M 30114) biomass (300 g/L) was produced by the fermentation process (bioreactor) for 27 runs (10 g/L biomass for each run), with a control sample, 200 mL of the YPG environment for preparing the biomass solution (4 g of peptone, 2 g of yeast extract, 4 g of G19 glucose, 200 mL of dH_2O), 10 g of sodium acetate ($C_2H_3NaO_2$), 200 mL of pure ethanol (C_2H_5OH), 30 mL of hydrochloric acid (HCl), 50 mL of chloroform ($CHCl_3$), 10 mL of isoamyl alcohol ($C_5H_{12}O$), 20 mL of acetic acid (CH_3COOH), and 20 mL of sodium hydroxide (NaOH). All the materials and implantation environments were made by the chemical and analytical grades of the Merck Company (Germany). Table 1 shows materials for mannoprotein extraction methods.

Methods

The production and purification of mannoproteins from yeasts typically involve several steps to extract and isolate these glycosylated proteins: yeast cultivation, inducing mannoprotein production, cell wall Disruption, cell debris removal, protein separation, characterization and quality control.

Preparing the fermentation process (Yeast culture and harvest)

A 30-L fermenter (Model Winpact FS01-V-B –L) was used in the fermentation process (Fig. 1). The H/D ratio of the vessel was 2.25, containing an agitator with three impellers, equipped with temperature, pH, oxygen concentration, and anti-foaming sensors with a control panel and display to control all yeast growth parameters. Four vertical pumps with changing current rates were available for changing the input and output currents during the fermentation. A rotating spiral kit was used for cell separation.

To achieve maximum growth rate of the yeast in the biotransformation process inside the fermenter and thus, the highest biomass of the grown yeast, 300 mL of an optimized Erlenmeyer environment cultured in perfectly optimized conditions in an Erlenmeyer in a shaker incubator was transferred to the 30-L fermenter (bioreactor) with 55.15 g/L of glucose, 9.35 g/L of the yeast extract with pH 4.99, and a fermentation time of 168 h [25]

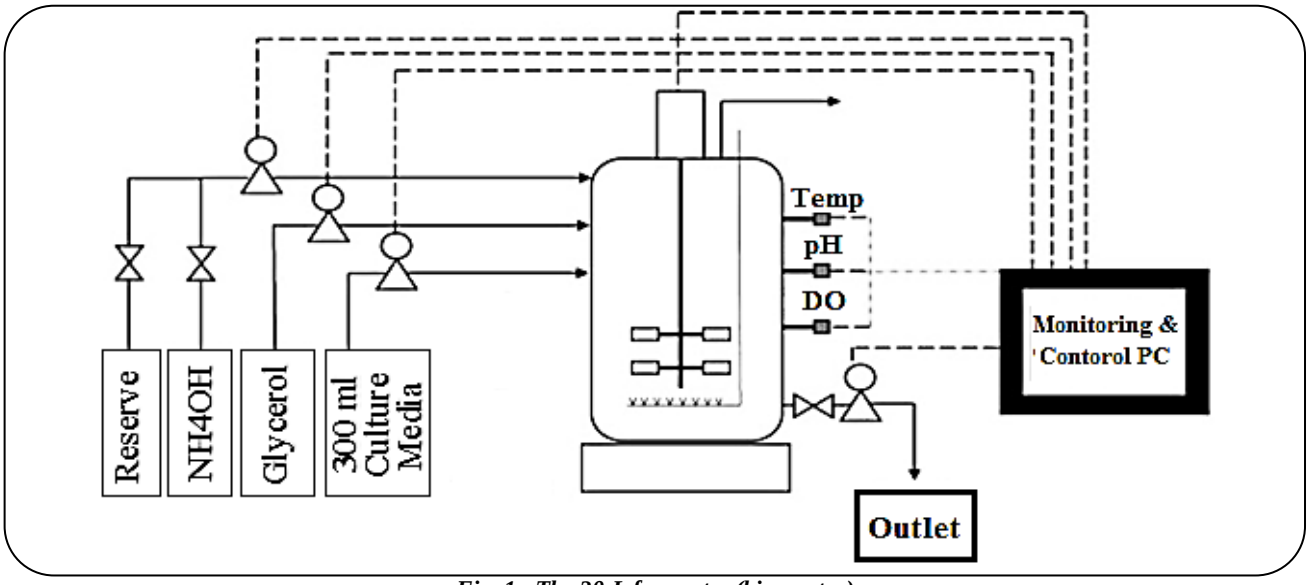

Fig. 1: The 30-L fermenter (bioreactor)

in a batch and non-continuous way. To reach the maximum yeast growth and prepare the highest yeast biomass, the initial conditions were set to DO calibration 100, 30 °C, inlet air volume 1 vvm^3, pH 4.7, and 500 rpm stirring speed. To find the optimal concentration of the biomass inside the fermenter with the maximum yeast growth, 30 mL of the sample fluid was weighed and transferred from the fermenter into a 10 mL falcon every 4 h for 30 h. This process was performed 10 times, each time in a 10 mL falcon, followed by centrifugation. The exact amounts of the parameters in all 10 samples are tabulated in the Results section.

Designing experiments (DOE) for the extraction of mannoprotein

Designing experiments (DOE) for the production and purification of mannoproteins from yeasts involves systematically varying factors to optimize the process. Homogenization, alkaline, and bio-emulsifier methods were performed to extract mannoprotein from the cell wall of *K. marxianus*. Designing and data modeling were conducted at three levels with various pHs, temperatures, and extraction methods by the QUALITEK-4 software, along with using the Taguchi method three times, with nine runs each time. For design, the three mentioned extraction methods were performed at 25, 30, and 35 °C and pH 2, 5, and 7. The experiments were designed by the Qualitek-4 software using the Taguchi method for three times and nine runs each time. The responses of each experimental design were calculated separately. In other words, the effect of each level of the extraction method on the three levels of temperature and pH was separately evaluated for three times of design. The goal is to systematically explore the factors that influence mannoprotein production and purification to achieve the highest yield and purity.

Preparing biomass before extraction (Wash the cells)

To separate and prepare the yeast cells, the YPG solution implantation environment containing 10 g/L biomass was centrifuged (4500 × g) in 10-50 mL falcons for 10 min. To homogenize the suspension, the cells were mixed with the highest intensity in a desktop vortex with 20-sec intervals in several rounds for 2 min. After separating and washing glass spheres, the obtained suspension was centrifuged at 4500 × g, and the precipitate was washed several times with cold deionized water.

Mannoprotein extraction by the bio-emulsifier method

The precipitate (10 g/L) containing the wall or cell biomass of the former biomass (20% w/w) in the buffer solution, 0.1 M sodium acetate, and 0.2 M acetic acid at pH 7 was autoclaved at 121 °C and 1 bar for 20 min. This was repeated with 10 g/L biomass in three runs with pH 2, 5, and 7 at 25, 30, and 37 °C. After centrifugation (4500 × g for 10 min), the mannoprotein of the solution was precipitated in three volumes of acidic ethanol. To complete the precipitation process, the sample was kept at 4 °C for 24 h

and then centrifuged at 8000 × g for 10 min. The spectrophotometry method was used to identify, characterize, and qualitatively measure the extracted mannoprotein. The bioemulsifier method is not a common approach for the extraction of mannoprotein*s* from yeast cells. However, this approach might not be as established or widely used as other extraction methods.

Mannoprotein extraction by the alkaline method

Cell biomass (10 g/L) and 100 mL of 0.2 N NaOH were combined, then maintained at 100 °C for two hours. The mixture was cooled, 1 N HCl was added to bring the pH down to 7, and then the mixture was centrifuged. For 10 g of the biomass, this procedure was done three times with pH values of 2, 5, and 7 at 25, 30, and 37 °C. Four times as much pure ethanol as the pellet volume was added. The mixture was centrifuged, the supernatant was removed, ethanol was added again, and the mixture was left to dry. The specttrophotometry method was used for qualitative measurement, identification, and characterization of the presence of the extracted mannoprotein. It's important to note that while the alkali method can effectively release mannoproteins, it might also lead to the extraction of other cellular components. In the extraction of mannoprotein by the alkaline method, it is better to concentrate the extracted mannoprotein using techniques such as ultrafiltration.

Mannoprotein extraction by the homogenization method

The biomass (10 g/L) was mixed with 20 mL of 100 mM acetate buffer (pH 4.5) and poured into a standard homogenization bottle, followed by setting the temperature. This was repeated for 10 g/L of the biomass in three runs with pH 2, 5, and 7 at 25, 30, and 37 °C. The solution was mixed at 12000 rpm and sonicated by a Brauns homogenizer in 8 cycles of 15 seconds (1-min intervals) for 2 min. The homogenized cells were examined under a microscope to ensure that they were fully broken. The cell residual was centrifuged at 4000 × g at 4 °C for 20 min. The specttrophotometry method was used for qualitative measurement, identification, and characterization of the presence of the extracted mannoprotein. The method might yield a mixture of various cellular components, so further purification steps could be necessary for obtaining highly pure mannoproteins. Optimization of homogenization parameters (pressure, number of passes, etc.) is crucial to achieving effective cell disruption without denaturing the proteins

Purification of extracted mannoproteins

In order to concentrate mannoprotein extracted by alkaline, homogenization, and bioemulsifier methods, the concentrated mannoprotein solution was washed with acetic acid (1%) in ethanol (96%) after being precipitated by centrifugation at 700 g for 10 min. The supernatant was maintained in incubation at 4 °C overnight and then centrifuged at 5000 g for 10 min to complete the precipitation.

The general process flow is shown in Fig. 2 for the preparation of mannoprotein-containing biomass from *Kluyoromyces Marcianus* yeast using a fermenter (bioreactor) and the extraction of mannoprotein using three distinct homogenization, alkaline, and bioemulsifier methods from the obtained biomass.

RESULTS AND DISCUSSIONS

Results and parameters of Kluyveromyces marxianus growth in the bioreactor (Yeast culture and harvest)

To prepare fermentation biomass from *K. marxianus*, the results and parameters obtained from the scale increase inside the fermenter (bioreactor) revealed that the maximum growth of the yeast was obtained during 30 h at optimal pH 4.6, 29 °C, 500 rpm, oxygen 19.7, inlet air, and DO 36.6, yielding an optimized biomass of 34.02 g/L for 3.5-L of the 30-L bioreactor volume. The fermentation process was stopped due to a sharp decrease in CO_2 after 30 hrs, indicating the end of the yeast growth cycle and the fermentation process inside the fermenter. Then, the fermentation of biomass by centrifugation began after removing the implementation environment of the fermentation fluid.

This experiment aimed to prepare and achieve maximum yeast growth and an optimized biomass concentration inside the fermenter. To achieve this aim, 30 mL of the sample fluid was transferred from the fermenter into a 10 mL falcon to determine the wet weight by centrifugation once every four hours within 30 h. This operation was performed 10 times, each time in a 10 mL falcon, followed by centrifugation. The factors and parameters in each of the 10 samples are listed in Table 2.

Table 2 represents the experimental design for the yeast growth under optimized conditions, the biomass concentration (30 mL in a falcon) taken from the fermenter in 30 h every 4-h, and wet weight. Fig. 3 shows biomass

Table 2: Results and parameters of Kluyveromyces marxianus growth in the fermenter (bioreactor)

NO.T	Time	Rpm	O_2	CO_2	pH	DO	T (°C)	Biomass (g/L)
1	12:30	500	20.3	0.05	4.31	32.5	27.3	4.12 ±0.05
2	18:00	500	19.7	0.15	4.66	29.1	28.9	6.53 ±0.05
3	20:30	500	19.5	0.21	4.59	25.8	29.3	9.22 ±0.05
4	22:30	500	19.3	0.25	4.51	20.9	29.4	12.56 ±0.05
5	00:30	500	19.5	0.23	4.56	26.5	29.1	16.31 ±0.05
6	2:30	500	19.4	0.24	4.57	25.8	29.2	18.53 ±0.05
7	4:00	500	19.5	0.26	4.57	26.7	29.1	21.64 ±0.05
8	6:30	500	19.5	0.24	4.58	31.2	29.1	26.1 ±0.05
9	8:00	500	19.6	0.23	4.61	32.7	28.9	28.2 ±0.05
10	10:30	500	19.6	0.21	4.60	33.2	28.9	31.5 ±0.05
11	12:00	500	19.1	0.13	4.60	35.2	29.2	33.2 ±0.05
12	14:30	500	19.7	0.6	4.60	36.6	29.1	34.02 ±0.05

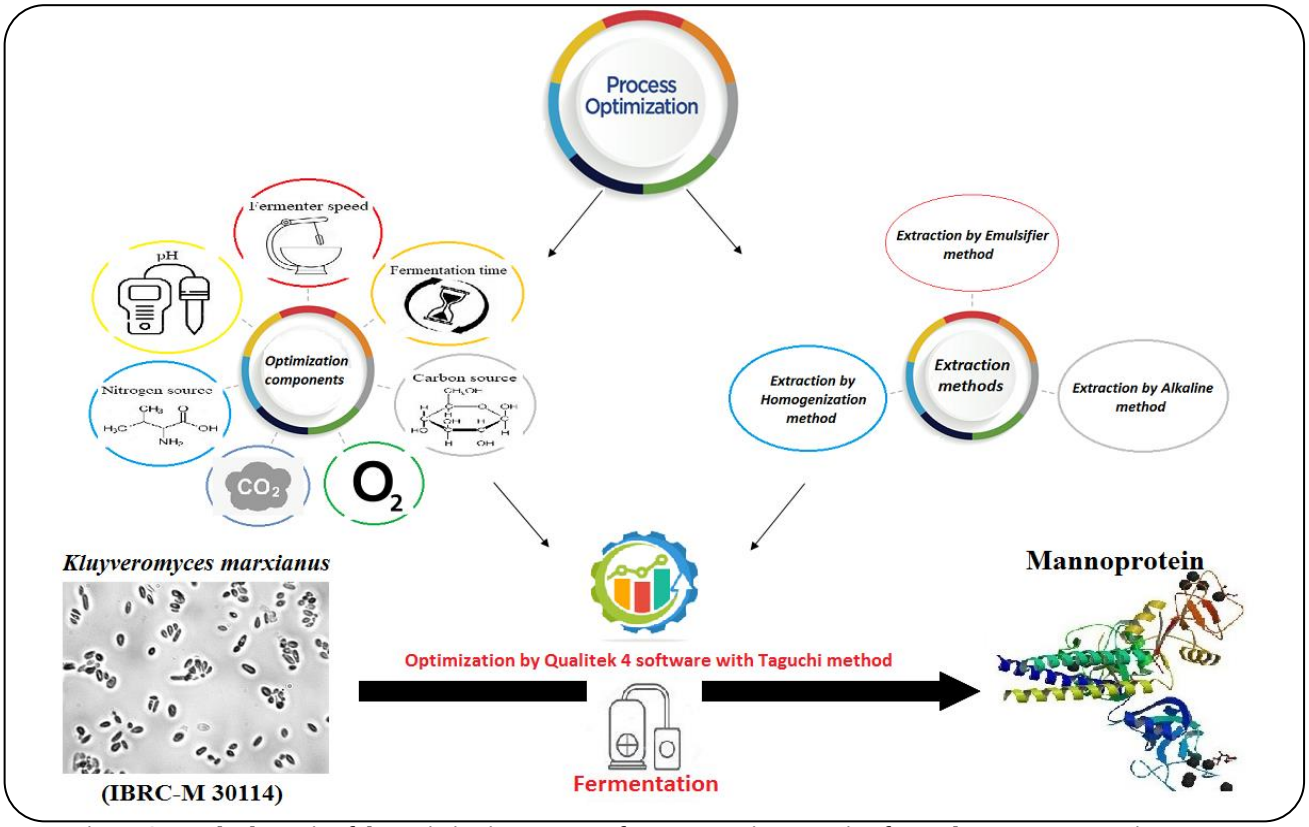

Fig. 2: General schematic of the optimization process of mannoprotein extraction from Kluyuromyces marcianos yeast

production in the batch and non-continuous fermentation systems within 30 h.

Predicting the true optimized response by Qualitek-4 software and the Taguchi method

Responses in each run of an experiment were introduced to the matrix table of the Taguchi experimental design to find the optimal conditions and predict the correct optimal response value. The responses were expressed in mg in 100 mL, and the first 9 samples were extracted for each 10 g/L yeast biomass. Figs. 4-6 show the maximum amount of the extracted mannoprotein in runs 4-6 and the minimum response in runs 1, 2, and 3, respectively (Tables 3-5).

Table 3: Mannoprotein (mg 100 m/L) for every 10 (g/L) of the yeast biomass extracted for the first 9 samples

NO.T	Acidity (pH)	Temperature (°C)	Extraction method	Mannoprotein (mg 100 m/L)
1	2	25	Bio-emulsifier	2.14
2	2	30	Alkaline	1.73
3	2	35	Homogenization	2.41
4	5	25	Alkaline	8.92
5	5	30	Homogenization	6.21
6	5	35	Bio-emulsifier	6.85
7	7	25	Homogenization	8.14
8	7	30	Bio-emulsifier	7.34
9	7	35	Alkaline	6.42

Table 4: Mannoprotein (mg 100 m/L) for every 10 (g/L) of yeast biomass extracted for second 9 samples

NO.T	Acidity (pH)	Temperature (°C)	Extraction method	Mannoprotein (mg 100 m/L)
1	2	25	Homogenization	2.42
2	2	30	Bio-emulsifier	2.11
3	2	35	Alkaline	1.74
4	5	25	Bio-emulsifier	6.81
5	5	30	Alkaline	8.90
6	5	35	Homogenization	6.22
7	7	25	Alkaline	6.40
8	7	30	Homogenization	8.11
9	7	35	Bio-emulsifier	7.31

Table 5:* Mannoprotein *(mg 100 m/L) for every 10 (g/L) of the yeast biomass extracted for the third 9 samples

NO.T	Acidity (pH)	Temperature (°C)	Extraction method	Mannoprotein (mg 100 m/L)
1	2	25	Alkaline	1.71
2	2	30	Homogenization	2.44
3	2	35	Bio-emulsifier	2.13
4	5	25	Homogenization	6.24
5	5	30	Bio-emulsifier	6.83
6	5	35	Alkaline	8.94
7	7	25	Bio-emulsifier	7.33
8	7	30	Alkaline	6.42
9	7	35	Homogenization	8.13

Comparing binary interactions for acidity, temperature, and the extraction method, and their effects on the response

Fig. 7 shows the responses for various temperatures and extraction methods. Each line in this diagram represents an extraction method at three temperature levels (25, 30, and 35 °C). Comparing the lines manifests that the highest response (8.9 mg 100 m/L) belongs to Level (1) 25 °C, the alkaline method and the lowest response (1.7 mg 100 m/L) belongs to Level (2) 30 °C, the alkaline method.

In Fig. 7, the horizontal axis shows the temperatures of 25 to 35 degrees at three levels, and the vertical axis shows the response value (manoprotein extraction rate). Each of the lines in the diagram shows one of the three methods of mannoprotein extraction separately for three temperature levels. By comparing these three conditions, it can be concluded that the temperature range between 25 and 35 degrees does not have a significant effect on the extracted mannoprotein, and only the acidity components

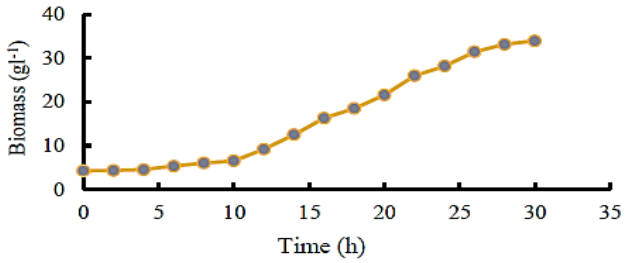

Fig. 3: Biomass production in the batch and non-continuous fermentation systems. The growth curve of Kluyveromyces marxianus in the fermenter (Bioreactor)

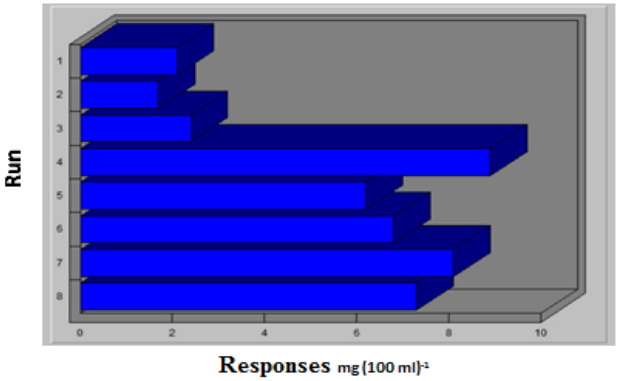

Fig. 4: The maximum amount of* mannoprotein *extracted in Run 4 and the minimum* mannoprotein *in Run 2

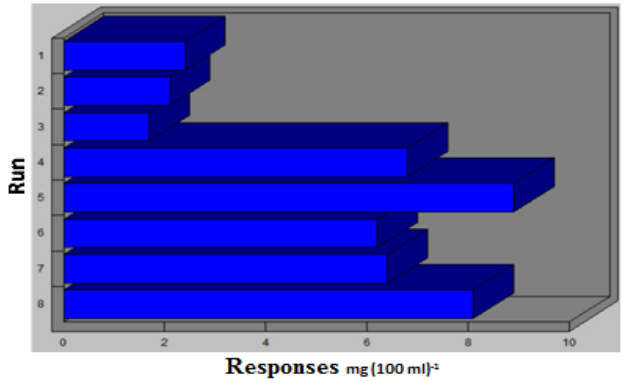

Fig. 5: The maximum amount of* mannoprotein *extracted in Run 5 and the minimum* mannoprotein *in Run 3

and the type of extraction method have an effect on the amount of mannoprotein obtained.

Fig. 8 displays the response values for various pHs and extraction methods. Each line in this diagram belongs to an extraction method compared at three pH levels (2, 5, and 7). It is obvious that the highest response value (8.9 mg 100 m/L) goes for Level (2), pH 5, the alkaline method, and the lowest response (1.7 mg 100 m/L) belongs to Level (1), pH 2, the alkaline method.

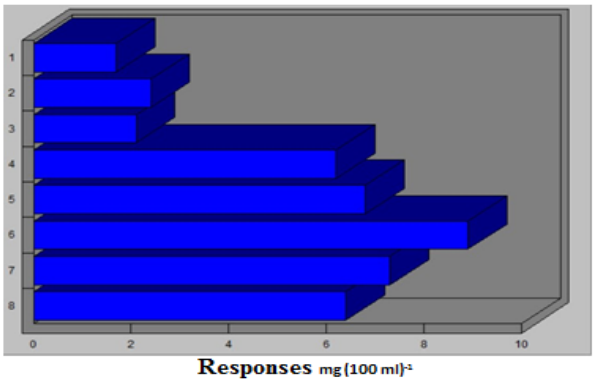

Fig. 6: The maximum amount of* mannoprotein *extracted in Run 6 and the minimum* mannoprotein *in Run 1

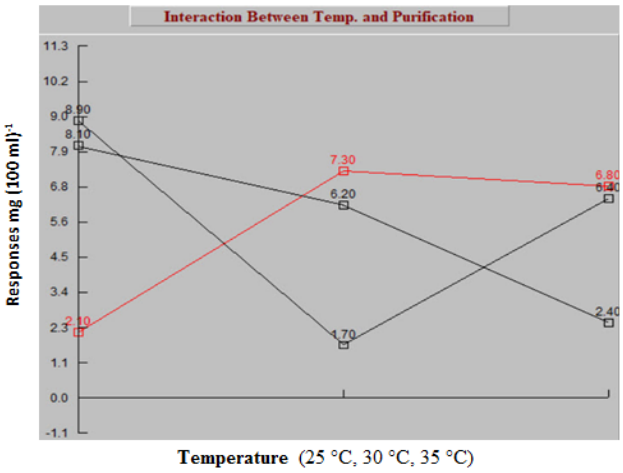

Fig. 7: Interactions of temperature and extraction methods, and their effects on the response value

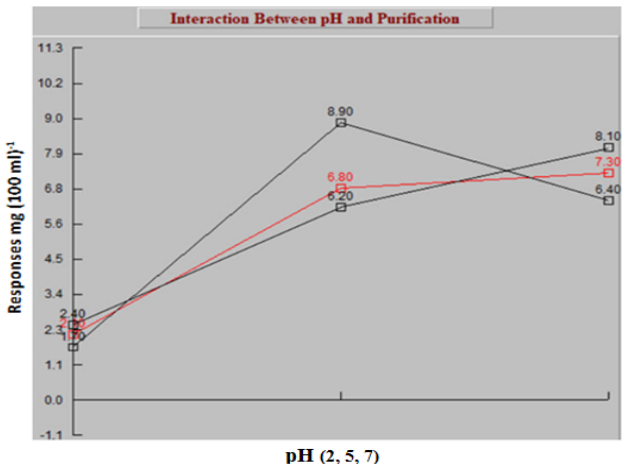

Fig. 8: Interactions of pH and extraction methods, and their effect on the response value

Fig. 9 shows the response values for various pHs and temperatures. Each line in this diagram belongs to a temperature

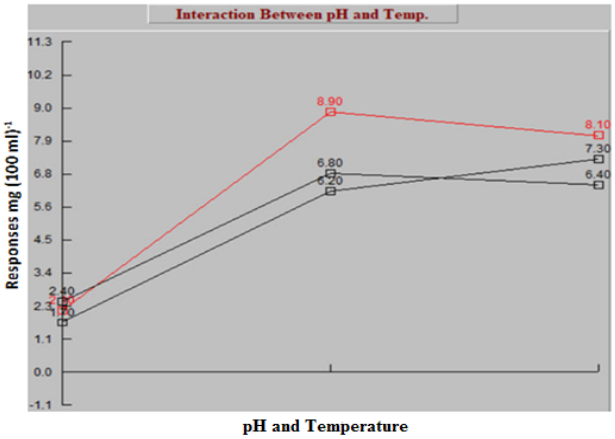

Fig. 9: Interactions of pH and temperature, and their effects on the response value

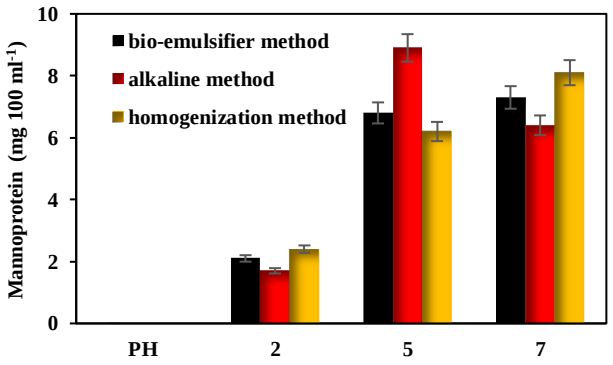

Fig. 10: Mannoprotein extraction with the bio-emulsifier, homogenization, and alkaline methods

effect compared at three pH levels (2, 5, and 7). The highest response value (8.9 mg 100 m/L) is displayed for Level (2) (pH 5, 25 °C), and the lowest response (1.7 mg 100 m/L) is shown for Level (1) (pH 2, 30 °C).

The results of mannoprotein extraction with the bio-emulsifier, homogenization, and alkaline methods in various pHs are illustrated in Fig. 10. The results showed that in acidities of 2 and 7, the homogenization method extracted the most mannoprotein from biomass, and in acidities of 5, the alkaline method extracted the most mannan. In other words, the homogenization method was more effective than the other two methods. And while it performed better in acids 2 and 7, it performed less efficiently than the other two acids at 5% acidity. Due to this, extraction by homogenization performed significantly better than extraction by alkaline and bioemulsifier methods.

To analyze the maximum amount of mannoprotein extraction, the results obtained by Taguchi software for the three designs (first to third) are show That The maximum amount of mannoprotein (8.243 mg 100 m/L) was extracted from every (10 g/L) yeast biomass at pH 5, 25 °C, and the alkaline method.

This suggests that the extraction of mannoprotein from biomass was not significantly affected by the temperature range of 25 to 35 degrees Celsius. It's important to note that while the alkali method can effectively release mannoproteins, it might also lead to the extraction of other cellular components. Further purification steps might be necessary to obtain highly pure mannoprotein samples. Additionally, optimizing the concentration of NaOH, treatment duration, and neutralization conditions is crucial to achieving the best results while minimizing damage to the extracted proteins.

Homogenization can efficiently disrupt yeast cells, leading to the release of intracellular components, including mannoproteins.The method might yield a mixture of various cellular components, so further purification steps could be necessary for obtaining highly pure mannoproteins. Optimization of homogenization parameters (pressure, number of passes, etc.) is crucial to achieving effective cell disruption without denaturing the proteins.

using a bioemulsifier method for mannoprotein extraction might require a good understanding of the properties of the bioemulsifier and its compatibility with yeast cells and mannoproteins. The method might not be as well-established as other extraction techniques, so careful optimization and testing are important to ensure effective and reliable results.

The goal of the current work was to better understand how *K. marxianus* yeast grows in a bioreactor and to develop an extraction procedure for mannoprotein, a bioemulsifier. The findings revealed important information on the development of *K. marxianus*, the techniques used for extracting it, and the amounts of mannoprotein extracted.

After 30 hours of fermentation at pH 4.6, 29 °C, and 500 rpm, the maximal biomass produced by *K. marxianus* in the bioreactor in terms of yeast growth was 34.02 (g/L). These findings are consistent with earlier research utilizing *K. marxianus* as the host organism and reporting substantial biomass output. In a fed-batch fermentation, *BEȘLIU et al.* [53] examined the development of *K. marxianus* and attained a biomass concentration of 10 g/L. Collectively, these results show that *K. marxianus*

has the capacity to produce biomass effectively. The alkaline approach at pH 5 produced the most mannoprotein 8.243 (mg/100 ml) from 10 (g/L) yeast biomass, amLording to the extraction techniques. This result is consistent with earlier research that extracted mannoprotein using alkaline techniques. For instance, *Li et al.* [54] used an alkaline extraction technique to extract Mannoproteins from *C. acuminata* seeds and obtained a yield of 46.9%. This shows that Mannoproteins may be extracted using the alkaline technique from a variety of sources, including yeast and plant-based materials.

The bioemulsifier technique also showed that (pH=5-7) was the ideal range for mannoprotein extraction. This result is in line with research on bio-emulsifier extraction from other sources. For instance in 2022, *Mallik* et al. examined the extraction of bio-emulsifiers from a microbial consortia and discovered that the extraction process was encouraged by a neutral pH range (pH=6-6.5) [55]. All of these results point to the critical role that pH levels play in maximizing the extraction of bio-emulsifiers, such as mannoproteins.

It is difficult to compare the amount of mannoprotein extracted in this study (8.243 mg/100 ml) with other studies because there aren't many direct comparisons available. It is important to keep in mind, though, that optimizing extraction variables like pH and temperature can have a big impact on how much mannoprotein is recovered. *Qiao et al*. utilized the alkaline technique for extracting Mannoproteins from fenugreek seeds and reported a yield of 5.9% [56]. These differences in extraction yield demonstrate how crucial it is to optimize extraction conditions in order to acquire the most mannoprotein possible.

It is critical to recognize the current study's limitations. The Taguchi technique and QUALITEK-4 software were utilized to optimize extraction procedures, however the study's main focus was on *K. marxianus* yeast growth and mannoprotein extraction from this particular yeast strain. The comprehension of extraction processes and the quantity of mannoprotein obtained would be further improved by comparisons with research employing different yeast strains or plant-based sources.

Studies have looked at numerous extraction techniques and sources to get Mannoproteins for diverse uses, expanding on the subject of mannoprotein extraction. For instance, Mannoproteins were isolated from Aloe vera leaves in research by *Ansari et al*. (2021) [57] using a mix of alkali and acid hydrolysis, producing a yield of 25.6%. Similar research was conducted by *Marathe et al*. (2019) [58] on the extraction of Mannoproteins from marine algae using an alkali-based technique, and they were sumLessful in obtaining a yield of 21.3%. These studies emphasize the potential of various sources for acquiring mannoproteins and show the adaptability of extraction techniques.

It's vital to take into amLount the differences in source materials and extraction methods when comparing the amount of mannoprotein isolated in this study (8.243 mg/100 m/L) with those extracted in previous studies. The yield of extracted Mannoproteins can be considerably impacted by the yeast strain selected, the growing environment, and the specific technology used. For instance, *Li et al*. (2018) [54] investigation on the extraction of Mannoproteins from yeast cell walls revealed an enzymatic extraction technique yield of 2.17%. These examples offer a wider context for assessing the yield of mannoprotein extraction, even if direct comparisons could be difficult due to variations in experimental settings.

It is also important to keep in mind that the structural makeup and functional characteristics of the isolated Mannoproteins might change. The functional properties and possible uses of yeast-based Mannoproteins, such as those from *Kluyveromyces marxianus*, can differ from those of plant-based Mannoproteins, such as those in Aloe vera or sea algae. Future research might focus on the structural characterisation of the isolated Mannoproteins and investigate their rheological behavior, emulsifying abilities, and compatibility with other formulations.

In our previous studies we have shown optimization of growth and purification of *Kluyveromyces marxianus* for mannan production in a lab scale, which can be used as a natural bioemulisifier [25]. In another study, our team reported the extraction of mannoproteins from this yeast cell wall and evaluated its functional properties to be used as an emulsifier in oil-in-water emulsions. The results of this study confirm that the yeast-derived mannoproteins are good at stabilizing these emulsions either in the presence or absence of different environmental conditions [45]. Recently we have optimized downstream process for extraction and purification of this mannoprotein complex in an attempt to obtain highly purified mannan oligosamLharides from *Kluyveromyces marxianus* in

Table 6. The highest amount of mannoprotein extracted in the best conditions

pH	The highest amount of extracted mannoprotein	Performance based on extraction percentage		
		Alkaline	Bioemulsifier	Homogenization
2	2.44 (mg 100 m/L) from each 10 (g/L) yeast biomass			✓
5	8.94 (mg 100 m/L) from each 10 (g/L) yeast biomass	✓		
7	8.14 (mg 100 m/L) from each 10 (g/L) yeast biomass			✓

laboratory scale [59]. So there was a great need to optimize the process in an industrial condition for a practical approach.

The novelty of our research topic "Design of Experiment for Mannoprotein Industrial-Scale Manufacturing Bio Process Optimization from *K. marxianus*" lies in several aspects:

Production point of view: Mannoproteins have various applications in the food, beverage, and pharmaceutical industries, including improving product stability, texture, and flavor. Optimizing the bio process for mannoprotein production at an industrial scale is a current challenge and opportunity.

DOE point of view: Utilizing DOE for optimization is a systematic and data-driven approach that allows for the efficient identification of key factors affecting the process. Applying this methodology to mannoprotein production from *K. marxianus* is a novel and effective way to enhance yields and quality.

Scale of production point of view: Focusing on industrial-scale manufacturing implies dealing with large volumes and the complexities of scaling up a process. This adds a unique dimension to the research, as it involves addressing real-world challenges that industries face.

Bioprocess engineering point of view: The research involves the field of bioprocess engineering, which combines biology and engineering principles to design and optimize processes for the production of valuable biological products. This interdisciplinary approach is an emerging and important area of study.

CONCLUSIONS

The growth of *K. marxianus* yeast in a bioreactor was sumLessfully explored in this work, and the extraction procedure for mannoprotein was improved. The findings shed important light on what circumstances are ideal for yeast growth and the extraction procedure. The constancy of utilizing alkaline techniques for mannoprotein extraction and the significance of pH settings are shown by comparisons with earlier work. The expertise of mannoprotein synthesis and extraction processes would increase with more study including a wider variety of yeast strains and plant-based sources, helping the development of applications for this bio-emulsifier in several industries.

Comparing the amounts of mannoprotein extracted by the bio-emulsifier, homogenization, and alkaline methods showed that the highest yield was obtained by the alkaline method. In the bio-emulsifier method, the maximum amount of mannoprotein was extracted at pH 7. At pH 5, the highest amount of mannoprotein was extracted by the alkaline method. In the homogenization method, the outcome of mannoprotein extraction was uppermost at pH 7.

It was found that temperatures at 25, 30, and 35 °C had no significant effects on the mannoprotein extraction yield, and only the interaction between pH and extraction methods determined the extracted mannoprotein levels. In general, the results showed that the homogenization method produced the maximum amount of mannoprotein extraction from biomass in acidities 2 and 7, whereas the alkaline approach produced the highest amount of mannoprotein extraction in acidity 5. The homogenization procedure is therefore superior to the other two methods. In acids 2 and 7, however, which are 5% lower than the other two acids, alkali and bioemulsifiers performed better and had higher efficacy. As a consequence, the homogenization method of extraction was more effective than the alkaline approach in terms of efficiency, while the alkaline method performed better than the other two methods of homogenization and bioemulsifier in terms of quantity. To analyze the maximum amount of mannoprotein extraction by the Taguchi method, the outcomes of Qualitek-4 software showed that the maximum amount of 8.243 (mg 100 m/L) from each 10 (g/L) yeast biomass was extracted at pH 5, 25 °C, and the alkaline method. Table 6 shows the highest amount of mannoprotein extracted in the best conditions.

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