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Optimization of Medium Components for Enhanced Expression of Recombinant Human Vascular Endothelial Growth Factor ($VEGF_{165}$) in *Kluyveromyces lactis* GG799

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Keywords
Vascular endothelial growth factor ($VEGF_{165}$), *Kluyveromyces lactis*, Biomass optimization, Recombinant protein, Response Surface Methodology

Abstract: Vascular endothelial growth factor ($VEGF_{165}$) is a vital biomarker linked to diverse physiological processes and pathological conditions like cancer, owing to its pivotal role in angiogenesis. Therefore, an efficient method to obtain recombinant $VEGF_{165}$ in high yield is required. This study focused on optimizing a medium to enhance the biomass of *Kluyveromyces lactis* (*K. lactis*) GG799 cells that express recombinant $VEGF_{165}$. Through screening various media components, galactose, tryptone, and yeast extract were identified as the most effective components. Using the Box–Behnken design, the medium components were optimized, resulting in a notable 31% increase in biomass concentration. The optimized medium, comprising 45 g L^{-1} galactose, 30 g L^{-1} tryptone, and 5 g L^{-1}yeast extract, achieved biomass productivity (P) of 0.26 gDCW L^{-1} h^{-1}. Additionally, the expression of $VEGF_{165}$ reached 1024.09 µg per L of the culture medium, representing a 1.27-fold increase compared to the unoptimized medium. These results imply that the proposed optimized medium can serve as a model for a process development strategy, providing a foundation for obtaining elevated yields of other recombinant proteins in *K. lactis*.

Rekombinant İnsan Vasküler Endotelyal Büyüme Faktörü ($VEGF_{165}$)'nün *Kluyveromyces lactis* GG799'da Geliştirilmiş Ekspresyonu İçin Ortam Bileşenlerinin Optimizasyonu

Anahtar Kelimeler
Vasküler endotelyal büyüme faktörü ($VEGF_{165}$), *Kluyveromyces lactis*, Biyokütle optimizasyonu, Rekombinant protein, Yanıt Yüzey Metodu

Öz: Vasküler endotelyal büyüme faktörü ($VEGF_{165}$), anjiyogenezdeki rolünden dolayı çeşitli fizyolojik süreçler ve kanser gibi patolojik hastalıklar ile ilişkili önemli bir biyobelirteçtir. Bu nedenle, yüksek verimde rekombinant $VEGF_{165}$ elde etmek için etkili bir yöntem gereklidir. Bu çalışmada, *Kluyveromyces lactis* (*K. lactis*) GG799 hücrelerinde rekombinant $VEGF_{165}$ ekspresyonunu artırmak amacıyla büyüme ortamının optimize edilmesi amaçlanmıştır. Farklı ortam bileşenlerinin taranması sonucunda galaktoz, tripton ve maya ekstraktı en etkili bileşenler olarak belirlenmiştir. Box-Behnken tasarımı kullanılarak seçilen ortam bileşenleri optimize edilmiş ve biyokütle konsantrasyonunda %31'lik kayda değer bir artış sağlanmıştır. 45 g L^{-1} galaktoz, 30 g L^{-1} tripton ve 5 g L^{-1} maya ekstraktı içeren optimize ortam ile 0,26 g_{DCW} L^{-1} h^{-1} biyokütle üretkenliğine (P) ulaşılmıştır. Ayrıca $VEGF_{165}$ ekspresyonu, optimize edilmemiş ortama kıyasla 1,27 kat artışla 1024,09 µg L^{-1} değerine ulaşmıştır. Elde edilen sonuçlar, önerilen optimize edilmiş ortamın *K. lactis*'te diğer rekombinant proteinlerin üretimine yönelik proses geliştirme stratejisi olarak bir model olabileceğini göstermektedir.

1. INTRODUCTION

Growth factors, which are biologically active molecules regulating cellular responses by binding to specific receptors on target cells, are essential for cellular regulation. Among these factors, Vascular Endothelial Growth Factor (VEGF) is a pivotal determinant, influencing both cell growth and survival, particularly in stimulating blood vessel formation through vasculogenesis and angiogenesis [1,2]. Although endothelial cells are the primary target of VEGF, its effects extend to various other cell types. VEGF plays a critical role in vascular development, contributing significantly to developmental processes and physiological homeostasis. Moreover, VEGF is vital for various physiological functions, including wound healing, regulation of the menstrual cycle, bone formation, hematopoiesis, and neural development [3–6]. Beyond its role in normal physiological processes, VEGF exerts pathogenic effects on tumor growth and metastasis by influencing vascular permeability and neoangiogenesis (neovascularization). Additionally, VEGF contributes to the progression of non-malignant diseases such as rheumatoid arthritis [7], psoriasis [8], diabetes, and neovascular eye diseases [9].

The VEGF family encompasses a group of proteins, including VEGF-A, B, C, D, E, and placenta growth factor (PIGF), regulated by three well-known receptors (VEGFR-1, VEGFR-2, and VEGFR-3) [10]. Initially identified as a vascular permeability factor (VPF) [11], VEGF-A, commonly referred to as VEGF, undergoes alternative splicing, resulting in various isoforms such as $VEGF_{165}$, $VEGF_{121}$, $VEGF_{189}$, and $VEGF_{206}$ in humans [12]. Among these isoforms, $VEGF_{165}$ holds particular significance and has been a focal point of extensive research, especially in the context of tumor development [13]. Functionally potent in tumor vascularization, $VEGF_{165}$ influences endothelial cell migration, proliferation, and tube formation. [14]. Structurally, $VEGF_{165}$ is a disulfide-linked homodimeric protein consisting of two monomers with a 165-amino acid polypeptide chain and a molecular weight of 38.2 kDa [15].

Recombinant DNA technology is a cornerstone of synthetic biology, particularly in the production of pharmaceutical proteins. The importance of recombinant therapeutic proteins in treating various diseases is steadily increasing, making them indispensable in the biotechnology market. Among the host systems utilized, yeasts are prominently featured for their numerous advantages in producing recombinant pharmaceuticals [16]. The main capacity of yeast is its sustained growth under straightforward media conditions in bioreactors. Yeasts excel in sustained growth under simple media conditions in bioreactors, and their amenability to genetic modification enhances their suitability as an ideal host system for pharmaceutical protein production [17]. *Kluyveromyces lactis* (*K. lactis*) emerges as one of the most promising yeast hosts for recombinant protein production due to its numerous advantages, including rapid growth, effective secretion levels, suitability for high cell density fermentation, low-cost culture media requirements, and ease of genetic manipulation compared to other yeast hosts. The industrial applications of *K. lactis* began with its use as a source of the enzyme lactase and later expanded into a protein supplement in foods. Notably, the production of bovine chymosin, the first recombinant protein from *K. lactis*, marked a significant milestone. Since then, numerous heterologous proteins have been recombinantly secreted from *K. lactis*. The GRAS (Generally Recognized as Safe) FDA status of these recombinant proteins underscores their safety and suitability for applications in both the food and pharmaceutical industries [18]. However, like all expression systems, certain process parameters must be optimized to ensure the successful expression of a protein in *K. lactis* and to increase production capacity before industrial-scale production for commercialization. For example, the impact of culture conditions and medium composition has been extensively studied to enhance the system's performance for higher biomass and protein titers. Previous studies have highlighted the crucial role played by medium components and their composition in both cell growth and protein expression levels [19–21].

As a result, medium optimization has emerged as a critical step in enhancing protein yield for industrial production. Traditional optimization, characterized by its time-consuming nature and neglecting factor interactions, has increasingly given way to statistical methods [22,23]. Response Surface Methodology (RSM) stands out as a valuable tool that integrates mathematical and statistical techniques to determine the optimal values of multiple variables in biochemical processes and has been widely adopted in recent years [24]. In this study, we aimed to optimize the medium components to maximize the biomass of *K. lactis*, thereby enhancing the expression of recombinant $VEGF_{165}$. *K. lactis* was selected as the ideal host organism due to its capacity to achieve high cell densities and consistent reproducibility at an industrial scale. Initial screening studies investigated the effect of various carbon, nitrogen, and extract sources on biomass production. Subsequently, the most effective components were optimized using RSM based on a full factorial central composite design (CCD).

2. MATERIAL AND METHOD

2.1. Reagents, Strains, and Growth Medium

All the chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Merck (Merck Millipore, Darmstadt, Germany). Ni-NTA agarose resin was purchased from Qiagen (Hilden, Germany), 1000 kDa MWCO cellulose acetate dialysis membrane was purchased from Spectrum Laboratories (California, USA), 3,3′,5,5′-tetramethylbenzidine (TMB) (Acros Organics, Belgium) and Pierce™ BCA Protein Assay Kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The ELISA washing buffer (PBST) consisted of 0.1 M PBS pH 7.2 containing 0.15M NaCl and 0.05% Tween 20, while the coating buffer was a 50 mM carbonate buffer, pH 9.6. The ELISA maxisorp immunoplate (96 flat-bottomed wells) was purchased

from Nunc (Roskilde, Denmark). Recombinant *K. lactis* strain GG799 (New England BioLabs, Massachusetts, USA) was previously constructed by inserting the human $VEGF_{165}$ cDNA (GenBank accession no. AF486837.1) into the *Xho*I and *Eco*RI sites of the pKLAC2 vector under the control of the *LAC4* promoter [25]. The recombinant strain was cultivated on YCB plates (3% 1 M Tris-HCl pH 7.4, 1.17% YCB, and 2% agar) containing 5 mM acetamide and preserved at −80 °C as a glycerol stock. Furthermore, YPLac (1% yeast extract, 2% peptone, and 4% lactose) (pH 7.4) served as the unoptimized medium for the expression performance of recombinant $VEGF_{165}$, allowing for comparison with the optimized medium. Optimization experiments at the shaking flask level were conducted in triplicate, and ELISA was performed in triplicate for each sample to determine the $VEGF_{165}$ concentration.

2.2. Expression and Purification of Recombinant Human $VEGF_{165}$

The inoculum was prepared by transferring a single colony of recombinant *K. lactis* GG799 from YCB plates into 25 mL of YPLac medium, followed by incubation at 30 °C at 250 rpm for 72 h. This pre-culture was then inoculated at a 1:100 ratio into a 2 L Erlenmeyer shaking flask containing 300 mL of YPLac for the $VEGF_{165}$ expression. After 72 hours of incubation, the supernatant containing the secreted recombinant $VEGF_{165}$ was collected by centrifugation at 5000 rpm for 5 minutes at room temperature to facilitate protein purification. For $VEGF_{165}$ purification, the supernatant was precipitated with a 60% ammonium sulfate solution in an ice bath for an hour, followed by centrifugation at 12000 rpm for 20 minutes. The protein pellet was then solubilized in 100 mM phosphate buffer (pH 7.4). Subsequently, a polycarbonate column containing Ni-NTA Agarose resin (Qiagen, Hilden, Germany) for affinity chromatography was washed with 100 mM phosphate buffer (pH 7.4). The protein solution was applied to the column, and the target protein was eluted from the column in fractions with 100 mM phosphate buffer containing 300 mM imidazole. The eluted $VEGF_{165}$ solution was dialyzed against 20 mM pH 7.4 HEPES buffer using a 1000 kDa MWCO cellulose acetate dialysis membrane (Spectrum Laboratories, California, USA) at +4˚C overnight on a magnetic stirrer. The resulting pure $VEGF_{165}$, obtained from the unoptimized medium, was used as the standard protein for the ELISA. The concentration of $VEGF_{165}$ was determined using the 'Pierce™ BCA Protein Assay Kit'.

2.3. Quantification of $VEGF_{165}$ by Direct ELISA

A direct ELISA protocol, adapted from methods outlined by Abcam (Cambridge, UK) with some modifications. $VEGF_{165}$, purified from recombinant *K. lactis* GG799, served as the standard protein solution, prepared as previously described. Samples and dilutions of the standards were prepared using a coating buffer. Maxisorp 96-well plates were coated overnight at 4°C with 100 µl $well^{-1}$ of either sample or standard. After four washes with 200 µL $well^{-1}$ of PBST, the wells were blocked with 100 µL of 2% w/v BSA in PBST for 2 h at room temperature. Following a rewashing step, the plates were incubated with 100 µL $well^{-1}$ of HRP-conjugated anti-6X His tag antibody (Cat. No. ab1187; Abcam, UK), diluted to 1:5000 in PBS, for 1 h at room temperature in the dark. After another round of washing, 100 µL of TMB substrate solution was added to each well for colorimetric development. The reaction was stopped by the addition of 25 µL $well^{-1}$ stop solution (4M H_2SO_4) and absorbances were measured at 450 nm using the Multiskan ™ FC Microplate Photometer (Thermo Fisher Scientific, USA). The $VEGF_{165}$ concentrations of the samples were calculated using the $VEGF_{165}$ standard curve following the manufacturer's protocol.

2.4. Growth Profile of *K. lactis* Cells

To assess the kinetics of $VEGF_{165}$ production, transformed *K. lactis* cells were inoculated into flasks containing 300 ml of YPLac (pH 7.4) medium and cultivated at 30°C with agitation at 250 rpm for 72 h. At predetermined intervals during the incubation, the biomass of *K. lactis* cells and the concentration of $VEGF_{165}$ were measured. Simultaneously, optical density, lactose consumption, and ethanol production were continually monitored. For dry cell weight (DCW) determination, 2 mL of culture broth samples were vacuum-filtered on pre-weighed filters (Whatman GF/C™). After two rinses with 5 mL distilled water, the filter papers were dried at 65°C for 24 hours and reweighed. Culture samples underwent ELISA analysis to quantify the secreted $VEGF_{165}$ protein. Lactose and ethanol levels in the culture medium were detected using the HPLC Dionex UltiMate 3000 system (Thermo Fisher Scientific, MA, USA) equipped with the HyperREZ XP Carbohydrate H^+ column.

2.5. Screening of Different Medium Components

To evaluate the impact of various medium components on the biomass production of recombinant *K. lactis*, cultures were conducted in 250 mL shake flasks containing 50 mL of the initial basal medium in triplicate. The basal medium composition mirrored that of YPLac, with each flask supplemented with 40 g L^{-1} of a carbon source, 20 g L^{-1} of a nitrogen source, and 10 g L^{-1} of an extract component. The cultures were then incubated for 72 hours at 30°C and 250 rpm. Eight carbon sources-lactose, glucose, starch, molasses, glycerol, acetic acid, sucrose, and galactose-were tested at an initial concentration of 40 g L^{-1}. Similarly, eight nitrogen sources-bacteriological peptone, meat peptone, casein peptone, urea, ammonium sulfate, diammonium sulfate, tryptone, and glycine-were examined at an initial concentration of 20 g L^{-1}. Additionally, three different extracts-yeast, malt, and meat extract-were chosen as additional nutritional sources and screened at an initial concentration of 10 g L^{-1}.

2.6. Box Behnken Design

Response surface methodology (RSM) was employed to optimize the medium components for the *K. lactis* cell biomass production. The experimental design utilized the Box-Behnken design within the Design Expert 13.0.0

program (Stat-Ease Inc., Minneapolis, USA). Overall, Box-Behnken design is characterized by a three-level second-order spherical design and offers several advantages compared to other designs. It allows the determination of significant factors using a limited number of runs and offers enhanced flexibility in selecting designs for a specified number of factors. In this study, the aim of using Box-Behnken design was to determine the optimal concentrations of galactose, tryptone, and yeast extract—identified as carbon, nitrogen, and extract sources, respectively—based on earlier screening.

Table 1. The central composite experimental design with coded values of variables.

Symbol	Independent variable name	Level		
		-1	0	1
A	Galactose (g L^{-1})	20	40	60
B	Tryptone (g L^{-1})	10	20	30
C	Yeast extract (g L^{-1})	5	10	15

A total of 17 experiments, encompassing all combinations of the three variables, were conducted with five replicates at the center point. This experimental setup aimed to fit a second-order polynomial model for each of the three independent variables, each at three levels. Additionally, the expression was carried out in 250 mL shake flasks filled with 100 mL of the medium in triplicate, incubated for 72 hours at 30°C and 250 rpm. Table 1 provides details on the normalized variables at levels of -1 (representing the lower value of the experimental conditions used), +1 (representing the higher value of the experimental conditions), and 0 (representing the central point condition) in the central composite design. For further clarity, Table 2 presents the experimental design with the terms in codes and their actual values within the specified range of variables.

Table 2. The central composite design of independent variables

Run	Coded value			Actual value		
	A	B	C	A	B	C
1	0	+1	+1	40	30	15
2	+1	-1	0	60	10	10
3	-1	0	-1	20	20	5
4	0	0	0	40	20	10
5	-1	0	-1	20	20	15
6	0	0	0	40	20	10
7	0	+1	-1	40	30	5
8	0	-1	+1	40	10	15
9	+1	0	+1	60	20	15
10	+1	+1	0	60	30	10
11	0	0	0	40	20	10
12	0	0	0	40	20	10
13	-1	-1	0	20	10	10
14	+1	0	-1	60	20	5
15	-1	+1	0	20	30	10
16	0	-1	-1	40	10	5
17	0	0	0	40	20	10

2.7. Statistical Analysis

The dry cell weight of the transformant *K. lactis* cells was taken as the response to be influenced by the four variables. The relationship between the response and the variables can be described by the second-order polynomial equation given as (1):

$$Y = \beta_0 + \sum \beta_i \chi_i + \sum \beta_{ij} \chi_i \chi_j + \sum \beta_{ii} \chi_i^2 \qquad (1)$$

where Y represents the response variable (in this case, the dry cell weight of the transformant *K. lactis* cells); β_0 represents the constant value; β_i, $\beta_{ij}$and β_{ii} represent the regression coefficients, χ_i and χ_j represent the independent variables in coded values. Analysis of variance (ANOVA) was used to obtain the second-order polynomial equation for all response variables. The significance of the model equation was assessed using the F-test. The quality of fit of the polynomial model equation was determined by the coefficient of determination (R^2) and adjusted R^2.

3. RESULTS

3.1. Process Kinetics of the Expression of $VEGF_{165}$ from *K. lactis*

The growth curve of *K. lactis* GG799 cultivated on YPLac medium with an initial lactose concentration of 40 g L^{-1} was monitored over 120 hours. Samples were collected at intervals during this duration. Figure 1 presents the time course of changes in key growth parameters, including dry cell weight, optical density, lactose consumption, and ethanol production in shake flask fermentation. The graph illustrates that the strain *K. lactis* GG799 efficiently utilizes lactose as a carbon source for growth, with rapid consumption observed. Moreover, the strain expresses the gene encoding $VEGF_{165}$ under the *LAC4* promoter, inducible by lactose [26]. The results suggest the strain's ability to utilize lactose for both growth and the expression of the target gene, showcasing its potential for biotechnological applications. Figure 1 also indicates that $VEGF_{165}$ production in shake flask cultures parallels cell growth until reaching the stationary phase at 72 hours. Concurrently, ethanol production peaks at 24 hours (2.49 g L^{-1}) and is nearly completely consumed. Notably, the culture exhibits a distinct exponential growth phase, with a maximum specific growth rate of 0.24 h^{-1} and a doubling time of 22 hours.

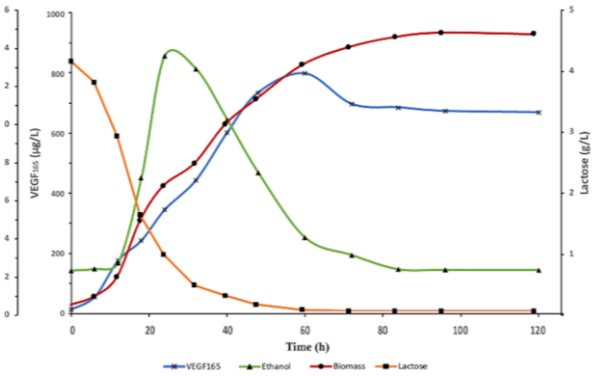

Figure 1. Production of recombinant $VEGF_{165}$ and cell growth profile of *K. lactis* GG799 in shake flask

3.2. Effect of Different Medium Components on Biomass and VEGF$_{165}$ Production

As an initial screening step before further optimization, various broth media in shake flasks containing different medium components were employed to investigate the effects on *K. lactis* cell growth and VEGF$_{165}$ expression. Figure 2 illustrates the impact of eight tested carbon sources added to the basal medium at 40 g L^{-1}. While glucose and glycerol exhibited favorable outcomes for biomass production, galactose emerged as a particularly notable carbon source, resulting in 1.58 ±0.013 µg L^{-1} of VEGF$_{165}$ production. Lactose and starch, also showed significant VEGF$_{165}$ production, yielding 0.99 ±0.011 and 0.83 ±0.014 µg L^{-1}, respectively. This result is consistent with many reports in the literature on recombinant protein production in *K. lactis* based on the use of galactose and lactose as carbon sources [27–29]. Indeed, galactose exhibited the highest biomass production among the tested carbon sources, yielding 15.98 ± 0.587 g L^{-1}. This was notably higher compared to both the control lacking any carbon source and the other carbon sources tested.

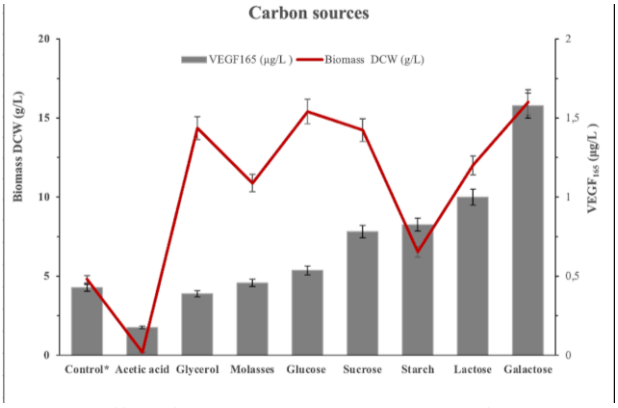

Figure 2. Effect of carbon sources on the biomass of *K. lactis* and VEGF$_{165}$ production. *Control culture without any carbon source

In this study, the influence of eight nitrogen sources on VEGF$_{165}$ production was investigated by supplementing them to a basal medium at a concentration of 20 g L^{-1}. The results, illustrated in Figure 3, revealed notable variations in both biomass and VEGF$_{165}$ production. Among the nitrogen sources examined, tryptone demonstrated the most favorable outcomes, yielding the highest biomass production at 16.18 ±0.213 g L^{-1}. Additionally, tryptone also resulted in the highest VEGF$_{165}$ production at 0.99 ±0.052 µg L^{-1}. Other nitrogen sources such as bacteriological peptone, casein peptone, and meat peptone showed similar VEGF$_{165}$ production levels, with yields of 0.86 ±0.019, 0.87 ±0.005, and 0.89 ±0.028 µg L^{-1}, respectively. These findings highlight the impact of different nitrogen sources on both biomass and VEGF$_{165}$ production, offering valuable insights for the optimization of culture conditions in *K. lactis* to enhance recombinant protein expression.

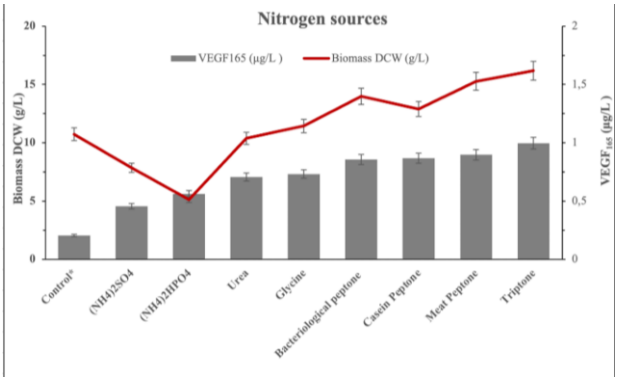

Figure 3. Effect of nitrogen sources on the biomass of *K. lactis* and VEGF$_{165}$ production. *Control culture without any nitrogen source

In this study, three extract components—malt, meat, and yeast extract—were evaluated as an additional nutritional resource in the growth medium to assess their effects on *K. lactis* biomass and VEGF$_{165}$ production. As depicted in Figure 4, the maximum VEGF$_{165}$ production of 0.68 ±0.017 µg L^{-1} and the highest biomass production of 16.18 ±0.758 g L^{-1}were achieved when yeast extract (10 g L^{-1}) was utilized. These results from the initial screening experiment highlight the significant impact of different medium components on both the biomass production of *K. lactis* and VEGF$_{165}$ production. Consequently, galactose, tryptone, and yeast extract were selected as the medium components for further optimization experiments.

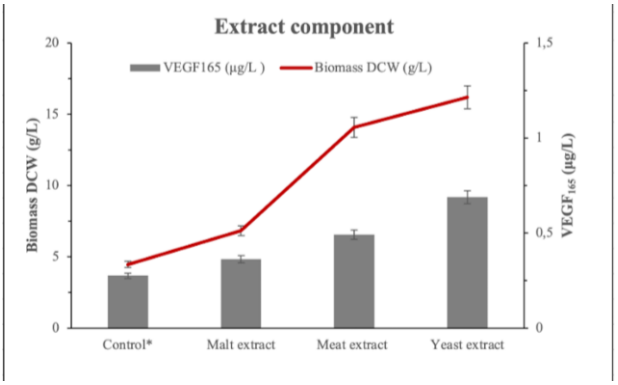

Figure 4. Effect of extract components on the biomass of *K. lactis* and VEGF$_{165}$ production. *Control culture without any extract component

3.3. Optimization of *K. lactis* Biomass using Response Surface Methodology

The objective of this study was to optimize the screened variables for biomass production to maximize VEGF$_{165}$ production, employing the Box-Behnken design. Experiments were conducted with compositions specified in Table 3. Following the screening experiments, three culture medium components—galactose, tryptone, and yeast extract—were selected for further evaluation in flask culture based on the experimental design. The relationship between the examined significant variables and biomass concentration, chosen as the response, was established using Response Surface Methodology (RSM). A central composite design (CCD) involving seventeen experiments, including 5 replicates of the central point, was executed. The observed and predicted responses, represented by dry cell weight values, for the 30

experiments are presented in Table 3. Utilizing the Box-Behnken experimental design, a wide range of biomass production in *K. lactis* GG799 was observed, ranging from 8.74 ± 0.22 to 17.52 ± 0.95 g L^{-1}. Comparing these experiments, the medium containing 20 g L^{-1} galactose, 10 g L^{-1} tryptone, and 10 g L^{-1} yeast extract exhibited the lowest biomass production at 8.74 g L^{-1}. Conversely, the highest biomass response of 17.52 g L^{-1} was achieved in a medium containing 40 g L^{-1} galactose, 20 g L^{-1} tryptone, and 10 g L^{-1} yeast extract.

Table 3. Central composite design and with actual and predicted response of biomass production with the independent variable values. Values are means of triplicates ± SD.

Run	Factors			Response Biomass (g L^{-1})	
	A	B	C	Actual	Predicted
1	40	30	15	17.12 ± 0.68	17.03
2	60	10	10	14.68 ± 0.60	14.55
3	20	20	5	11.61 ± 1.04	11.25
4	40	20	10	16.33 ± 0.45	15.86
5	20	20	15	11.30 ± 0.08	11.26
6	40	20	10	17.52 ± 0.95	15.86
7	40	30	5	15.94 ± 0.37	16.17
8	40	10	15	15.04 ± 0.50	14.81
9	60	20	15	17.18 ± 0.42	17.54
10	60	30	10	16.94 ± 1.50	16.67
11	40	20	10	15.87 ± 0.61	15.86
12	40	20	10	14.97 ± 0.75	15.86
13	20	10	10	8.74 ± 0.22	9.01
14	60	20	5	15.08 ± 1.00	15.11
15	20	30	10	11.93 ± 1.07	12.06
16	40	10	5	13.14 ± 0.4	13.23
17	40	20	10	14.63 ± 0.67	15.86

By applying the response surface, the following second-order regression equation explained the biomass as the interactions of the three culture medium components were yielded and is presented in the following equation:

$$Y_{biomass} = 15,86 + 2,54A + 1,29B + 0,6096C - 0,2330AB + 0,6035AC - 0,1813BC - 2,15A^2 - 0,6348B^2 + 0,0840C^2 \quad (2)$$

where Y is the response (biomass) and; A, B, and C are galactose, tryptone, and yeast extract respectively. Plus (+) and minus (-) symbols represent positive and negative effects on the response, respectively.

The effect of three independent factors on *K. lactis* GG799 biomass production was assessed using the analysis of variance (ANOVA) as detailed in Table 4. F-test, comparing the mean square regression to the mean square residual (12.19), indicates the significance of the process model with $p<0.05$. The lack of fit F-value of 0.15 means the lack of fit is not significant relative to pure error. The goodness of fit of the model is further evaluated using the R^2 coefficient (R^2 value), which stands at 0.94, explaining 94% of the validity of the response. Additionally, the predicted R^2 of 0.8207 is in reasonable agreement with the adjusted R^2 of 0.8629.

Among the terms analyzed, A (galactose), B (tryptone), and A^2 (galactose squared) with p-values less than 0.05 are deemed significant model terms. In Table 4, it is evident that galactose and tryptone exerted a more substantial effect on biomass production, as indicated by their relatively high mean square values (51.55 and 13.33 g L^{-1}, respectively), compared to yeast extract (2.97 g L^{-1}). Specifically, galactose played a crucial role in inducing heterologous protein production and enhancing cell growth through the utilization of the *LAC4* promoter (P_{LAC4}). This promoter drives the expression of the *LAC4* gene, encoding native lactase (β-galactosidase). β-galactosidase is an integral component of the lactose-galactose regulon, enabling the organism to efficiently use lactose as a carbon and energy source [30].

Table 4. Analysis of variance results for the model for biomass production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	91.58	9	10.18	12.19	0.0017	Significant
A (galactose, g L^{-1})	51.55	1	51.55	61.74	0.0001	
B (tryptone, g L^{-1})	13.33	1	13.33	15.97	0.0052	
C (yeast extract, g L^{-1})	2.97	1	2.97	3.56	0.1011	
AB	0.22	1	0.22	0.26	0.6257	
AC	1.46	1	1.46	1.74	0.2281	
BC	0.13	1	0.13	0.16	0.7033	
A^2	19.54	1	19.54	23.41	0.0019	
B^2	1.70	1	1.70	2.03	0.1970	
C^2	0.03	1	0.03	0.036	0.8557	
Residual	5.84	7	0.83			
Lack of Fit	0.58	3	0.19	0.15	0.9279	Not significant
Pure Error	5.27	4	1.32			
Cor Total	97.43	16				

R^2=0.94, Adjusted R^2=0.8629, Prediction R^2=0.8207, Adeq Precision=12.1756

Biomass production was predicted for various values of the tested variables, and the results are illustrated through three-dimensional response surface plots (Figure 5). The plots depict the response (biomass production) on the z-axis against any two independent variables. In Figure 5a, the 3D plot showcases the effects of galactose and tryptone concentrations on biomass production. The plot suggests that intermediate concentrations of galactose and tryptone result in a more pronounced increase in biomass production. Figure 5b highlights the interaction between galactose and yeast extract. It becomes evident that at higher concentrations of both components, biomass production can be elevated. In Figure 5c, the plot reveals the relationship between yeast extract and tryptone concentrations, indicating that increased biomass is observed with the rising concentration of yeast extract and tryptone. These three-dimensional response surface plots offer valuable insights into the intricate relationships among the variables and their impact on biomass production.

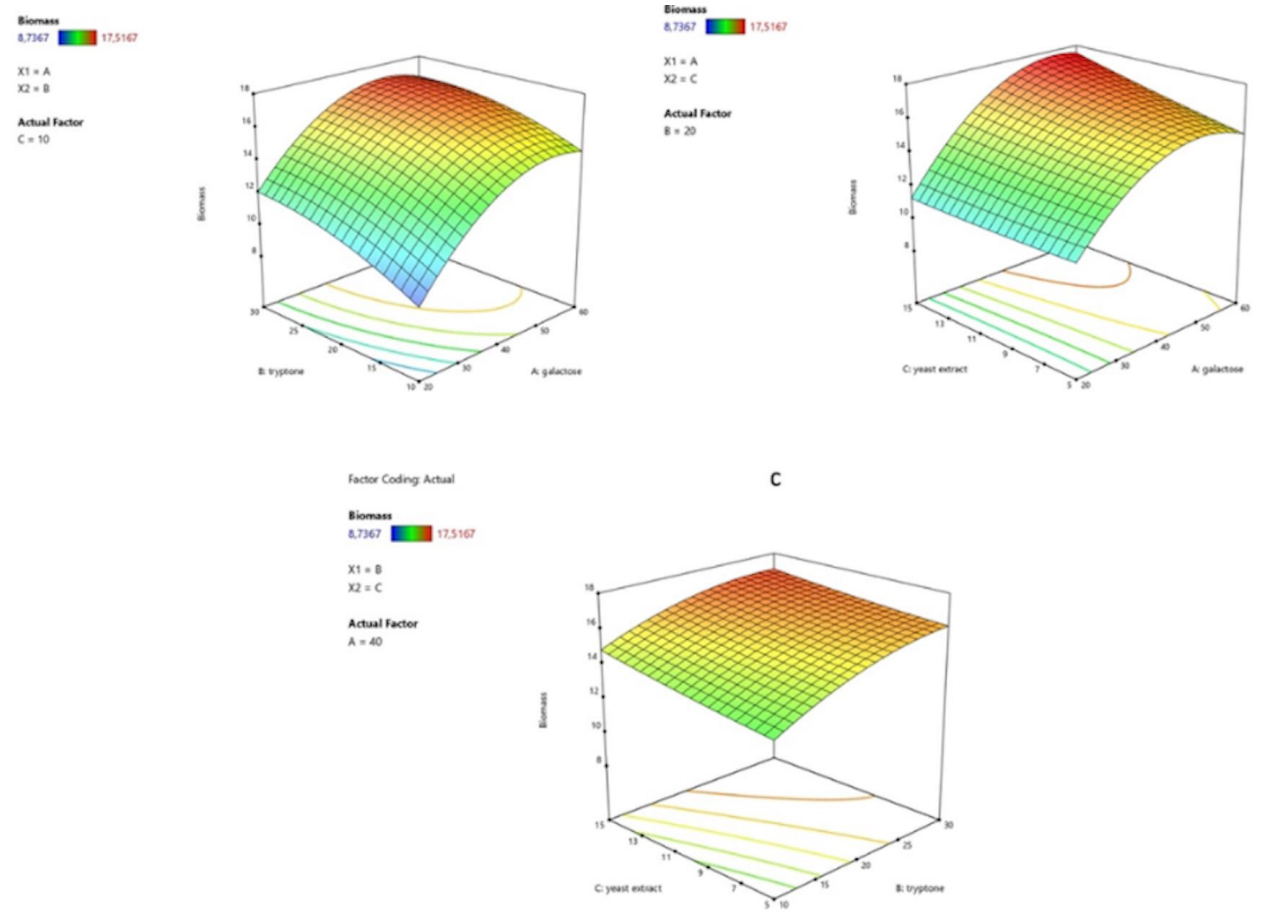

Figure 5. Three-dimensional response surface plot for *K. lactis* GG799 biomass production showing the interactive effects of (a) galactose and tryptone, (b) galactose and yeast extract, and (c) yeast extract and tryptone with the remaining factors held constant at the middle level of the Box-Behnken experimental design.

3.4. Model Diagnostics

To assess the adequacy of the model, the difference between observed values and calculated values obtained by the model, termed 'residuals,' was examined. The normal probability plot of 'studentized' residuals emerged as a crucial diagnostic tool for identifying and understanding systematic deviations from assumptions that errors are normally distributed and independent of each other [31]. As depicted in Figure 6, the graphical representation of residuals as a function of estimated values reveals that the errors of the biomass production models are scattered close to the diagonal line. This pattern implies that the model is a good fit and that the errors are normally distributed. The normal probability plot serves as a valuable validation tool, affirming the reliability of the model for predicting biomass production.

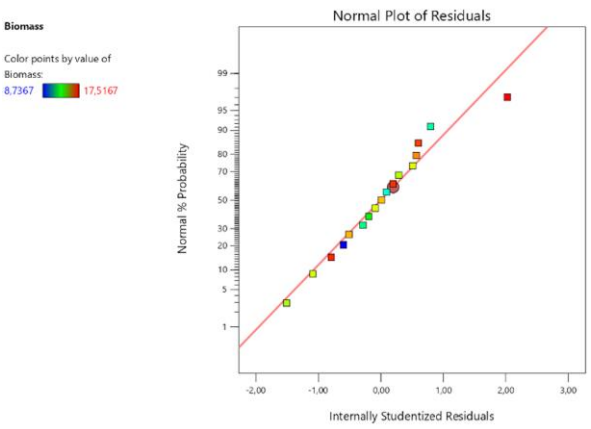

Figure 6. Normal (%) probability plot of the 'studentized' residuals for the model of biomass production

3.5. Validation of the Model

The model was optimized using the 'Point Optimization' of the Design Expert to achieve the maximum biomass production of *K. lactis* for recombinant $VEGF_{165}$. To validate the optimization, the criteria for biomass were set to 'maximize,' while galactose and tryptone were set to 'in range,' and yeast extract was set to 'minimize.' The level of yeast extract was minimized because it was identified as a non-significant model term with a p-value greater than 0.1. To verify the model, duplicate experiments were performed at the proposed optimum levels of the medium, comprising 45 g L^{-1} galactose, 30 g L^{-1} tryptone, and 5 g L^{-1} yeast extract. The observed value for the biomass production was 18,65 g L^{-1} and the predicted mean was 16.46. The 95% prediction interval ranged from 14.02 to 18.90. Additionally, in the same study, $VEGF_{165}$ concentration increased from 803.63 µg L^{-1} to 1024.09 µg L^{-1} after optimization, representing a 1.27-fold improvement. The biomass productivity (P), expressed in g of dry biomass per L of culture medium per h of cultivation time, reached 0.26 (g_{DCW} L^{-1} h^{-1}) at the optimal levels. The excellent agreement between predicted and experimental biomass production after optimization reaffirms the validity of the screened medium components under the Box-Behnken experimental design.

4. DISCUSSION

The optimization of biomass production in *K. lactis* is paramount from an industrial perspective, as it significantly enhances the efficiency and feasibility of the processes involving recombinant protein production and other biotechnological applications. Numerous studies in the existing literature have emphasized maximizing *K. lactis* biomass production due to its inherent advantages, including, faster and cost-efficient recombinant protein production, scale-up potential, and enhanced productivity in terms of protein expression. This study delves into the impact of various growth medium components on the biomass production of *K. lactis* GG799. Subsequently, a statistical optimization approach was employed to augment the efficacy of the most impactful components.

Initially, the growth curve of *K. lactis* GG799 was studied over 120 hours, analyzing the key parameters, including dry cell weight, optical density, lactose consumption, and ethanol production in the growth medium YPLac, which includes lactose as a carbon source. Rapid lactose consumption was observed, facilitating both growth and the expression of the target gene $VEGF_{165}$ under the *LAC4* promoter (P_{LAC4}). The P_{LAC4} encodes native lactase, an essential part of the lactose-galactose regulon, enabling *K. lactis* to utilize lactose as a carbon and energy source [32]. Biomass production and $VEGF_{165}$ expression progressed in parallel progress until reaching the stationary phase at 72 hours. Subsequently, while the biomass continued to increase, the $VEGF_{165}$ expression remained constant. Within the initial 24 hours, most of the carbon source was consumed, and ethanol production peaked, gradually diminishing as ethanol was utilized as a substrate through the respire-fermentative metabolism of *K. lactis* [33]. The culture exhibited a distinct exponential growth phase, resulting in a maximum specific growth rate of 0.24 h^{-1} and a doubling time of 22 hours. This growth rate closely aligns with the 0.29 h^{-1} reported by Toivari et al. (2013) for the same strain [34] and slightly exceeds the 0.16 h^{-1} reported by Hun et al. (2013) for another strain (*K. lactis* NRRL Y-110) [27]. Furthermore, Boender et al. (2009) noted that a doubling time of 23.1 hours represents rapid growth for yeast in many natural environments [35].

Most studies on *K. lactis* growth have utilized complex media or glucose as a primary carbon source. However, this study diverges by screening eight carbon sources to determine the most effective components for biomass production and $VEGF_{165}$ expression. Both lactose and galactose serve as sugars capable of inducing expression under the *LAC4* promoter in *K. lactis*. It was reported that the transcription of *LAC4* can be induced 100-fold in the presence of lactose or galactose [36]. The screening assay highlights galactose and lactose stand out as carbon sources, yielding the highest expression of $VEGF_{165}$. Interestingly, the use of galactose notably enhances $VEGF_{165}$ production per unit of biomass compared to lactose. This suggests galactose supplementation exerts a more pronounced impact on the efficiency of $VEGF_{165}$ expression.

In the unoptimized medium, two nitrogen sources, peptone and yeast extract are present. To enhance biomass production and $VEGF_{165}$ expression, various nitrogen sources and extract components were screened. Among the nitrogen sources investigated tryptone yielded the most favorable outcomes, resulting in the highest biomass and $VEGF_{165}$ production, following bacteriological peptone and meat peptone. This suggests that in the metabolic process of tryptone in *K. lactis* GG799, utilization of the peptides and amino acids present in tryptone serves as the most effective nitrogen source, promoting growth and biomass production. Moreover, among the diverse extract components tested, yeast extract, rich in amino acids, peptides, vitamins, and minerals, emerged as the most potent supporter of *K. lactis* GG799 cell growth and proliferation.

This study aimed to optimize the screened variables for enhanced biomass production, with the ultimate goal of maximizing $VEGF_{165}$ production, employing the Box-Behnken design. The relationship between the three examined variables (galactose, tryptone, and yeast extract) and biomass concentration, chosen as the response, was established using RSM. The maximum biomass response of 17.52 g L^{-1} was achieved in a medium comprising 40 g L^{-1} galactose, 20 g L^{-1} tryptone, and 10 g L^{-1} yeast extract. The wide variation in biomass production, ranging from 8.74 ± 0.22 to 17.52 ± 0.95 g L^{-1}, underscores the significant influence of factors in recombinant protein production [37, 38]. The impact of the three independent factors on *K. lactis* GG799 biomass production was evaluated through ANOVA, revealing that the interaction between the variables significantly contributes to improving biomass production ($p<0.05$). Furthermore, the difference between the predicted and adjusted R2 was less than 0.2, as expected for the fit statistic [39]. According to ANOVA, of the terms examined, A (galactose), B

(tryptone), and A2 (galactose squared) with p-values less than 0.05 are considered significant model terms. Galactose and tryptone had a more pronounced impact on biomass production, as reflected by their relatively high mean square compared to yeast extract. Additionally, the 3D plot illustrates the effects of galactose and tryptone concentrations on biomass production, suggesting that intermediate concentrations of galactose and tryptone lead to a more notable increase in biomass production. Also, the graphical representation of residuals as a function of estimated values indicates that the errors adhere to a normal distribution, affirming the good fit of the proposed model. The positive and negative contributions of the coefficients signify that any increase in medium components results in a statistically significant decrease or increase in biomass production, respectively. In equation (1), it is observed that the supplementation of galactose, tryptone, and yeast extract has a positive effect. The combined interaction of galactose and yeast extract (AC) has a positive effect on biomass production, while those of galactose and tryptone (AB) and tryptone and yeast extract (BC) are positive. However, the quadratic contribution of galactose and tryptone has a negative effect, while yeast extract shows a positive effect.

Under the experimental conditions considered in this study, the optimal medium composition predicted by Design Expert software was determined to be 45 g L^{-1} galactose, 30 g L^{-1} tryptone, and 5 g L^{-1} yeast extract. This resulted in biomass production of 18.65 g L^{-1} and $VEGF_{165}$ production of 1024.09 µg L^{-1}. Notably, these values are approximately 1.31 and 1.27-fold higher than values obtained under unoptimized conditions, respectively. Despite the different medium compositions, the maximum biomass production obtained in this study (18.65 g L^{-1}) exceeded the values reported in the literature. For instance, Pandey and Veeranki [21] studied the optimization of human interferon-gamma by varying growth parameters and carbon source concentration and they observed the maximum biomass of *K. lactis* GG799 (15 g L^{-1} dry cell weight) produced at a lactose concentration. In another study, Pandey et al. optimized the medium components for maximizing the biomass of recombinant *K. lactis*, resulting in a biomass production increased to 23.1 g L^{-1}, which was 1.5-fold higher as compared to the initial biomass value observed [20]. Zaharah et al. [38], developed a medium for recombinant endo-β-1,4- xylanase (Xyn2) production using *K. lactis* GG799, and the designed medium improved biomass output (5.67 g L^{-1}) and protein production by approximately 9 and 22%, respectively. Finally, Hun et al. investigated the effect of medium composition on the production of probiotic *K. lactis* NRRL Y-110 biomass, with maximal biomass of 6.32 g L^{-1} yield obtained in shake flask cultivation [27].

Table 5. Comparison of results before and after optimization

Variable	Un-optimized medium		Optimized medium	
	g L^{-1}		g L^{-1}	
Medium components	Lactose	40	Galactose	45
	Peptone	20	Tryptone	30
	Yeast extract	10	Yeast extract	5
Biomass (g L^{-1})		14.22		18.65
$VEGF_{165}$ (µg L^{-1})		803.63		1024.09
Biomass productivity (g_{DCW} L^{-1} h^{-1})		0.19		0.26

5. CONCLUSION

In this study, we significantly enhanced the expression of recombinant human $VEGF_{165}$ by optimizing the biomass production of *K. lactis* through a statistical experimental design. Among the screened carbon, nitrogen, and extract components, galactose, tryptone, and yeast extract emerged as key factors leading to higher biomass production and increased $VEGF_{165}$ expression. Through the application of the Box-Behnken design, we achieved a remarkable increase in biomass production at the shake flask level compared to the initial production in the unoptimized growth medium. Concurrently, the expression level of recombinant $VEGF_{165}$ experienced a significant increase, surpassing 1 mg L^{-1} with the optimized medium. These findings underscore the significant influence of medium components on both the growth of *K. lactis* GG799 and the production of recombinant $VEGF_{165}$. Nonetheless, the insights gained from this research have broader implications, offering potential relevance for the overexpression of other recombinant proteins utilized in various biotechnological applications. Furthermore, studies employing optimized media will advance the current understanding of the mechanism of heterologous protein secretion by *K. lactis* and facilitate new strategies to enhance the production capabilities of these cells.

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