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Valorization of Dairy Sludge as a Low-Cost Substrate for Enhanced Biomass and Exopolysaccharide Production by *Enterococcus durans* K48

N. Taheri Nasab[1], M.R. Edalatian Dovom[1]*, M. Moeenfard[1], L. Roozbeh Nasiraei[2]
1- Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran
(*- Corresponding Author Email: edalatian@um.ac.ir)
2- Department of Food Science and Technology, No.C., Islamic Azad University, Nour, Iran

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Abstract

Dairy industry waste, particularly organic-rich dairy sludge, represents a promising and sustainable resource for biotechnological applications. This study evaluated the potential of dairy sludge as a cost-effective medium for the co-production of biomass and exopolysaccharides (EPS) by selected Enterococcus strains. The physicochemical parameters of the dairy sludge were characterized, revealing a composition of 32.70% fat, 29.32% protein, 9.56% ash, and 30.17% carbohydrates (which adjusted to 28.50% after spray-drying). Five strains of Enterococcus were screened through a quantitative culture-dependent approach to evaluate their growth and EPS production. Among the tested isolates, *Enterococcus durans* K48 exhibited the highest production potential and was selected for further study. Response Surface Methodology (RSM) was employed to optimize the concentrations of key medium components: dairy sludge, sucrose, and yeast extract. Under the optimized conditions (15% dairy sludge, 5% sucrose, and 2.5% yeast extract), a maximum biomass yield of 8,440 mg/L and an EPS yield of 394.2 mg/L were achieved. These findings demonstrate that dairy sludge can serve as a valuable source and sustainable substrate for Enterococcus cultivation, facilitating efficient biomass and EPS production while contributing to the valorization of dairy processing waste.

Keywords: Biomass, Dairy sludge, Lactic acid bacteria, Medium optimization, Valorization

Introduction

Dairy industries are among the main producers of organic waste within the food processing sector. The sludge generated from their wastewater treatment systems possesses considerable biotechnological potential due to its high organic load and its content of lactose, proteins, and micronutrients (Shete & Shinkar, 2013; Ryan & Walsh, 2016). One of the primary components of these effluents is dairy sludge, originating from processes such as separation and bactofugation (McCarthy, 2011). Effective management and value-added utilization of this waste stream could not only reduce the environmental impacts associated with disposal, but also facilitate the recovery of high-value biological compounds (Shete & Shinkar, 2013). In recent years, the use of dairy sludge as a fermentation medium for producing bioactive compounds has gained growing interest, however, its efficiency as a targeted substrate for exopolysaccharide (EPS) production remains unexplored.

EPS are high-molecular-weight polymers secreted by microorganisms into their surrounding environment (Wang *et al.*, 2023).

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Owing to their rheological, functional, and bioactive properties, EPS hold significant relevance for food, pharmaceutical, and biotechnological industries (Naykodi, Doriya, & Thorat, 2025). Although conventional EPS production commonly depends on costly culture media, recent strategies focus on utilizing low-cost raw materials and food wastes—such as cheese whey, molasses, and agricultural by-products—to improve economic feasibility (Sakr, Massoud, & Ragaee, 2021). Lactic acid bacteria (LAB) are widely recognized for their EPS-producing potential (Surayot *et al.*, 2014). In particular, the genus Enterococcus, which naturally inhabits dairy environments, exhibits promising EPS synthesis capabilities with diverse structural and functional characteristics (Gaur & Annapure, 2025).

Despite these advantages, limited information is available regarding the performance of Enterococcus strains in unconventional, nutrient-rich substrates such as dairy sludge. Moreover, the relationship between sludge composition, strain metabolism, and EPS productivity remains poorly understood. This gap highlights the need for systematic investigation into the suitability of dairy sludge as a fermentation medium for EPS-producing Enterococcus strains.

To Address this research gap, the present study aimed to investigate the feasibility of utilizing dairy sludge as a sustainable and low-cost substrate for microbial biomass and exopolysaccharide (EPS) production. Five Enterococcus strains isolated from traditional dairy products were screened for their biomass-forming ability and EPS production, and the most promising strain (*Enterococcus durans* K48) was selected for further investigation. Biomass production was subsequently optimized using Response Surface Methodology (RSM). The novelty of this study lies in demonstrating the potential of dairy sludge, an underutilized dairy by-product, as an alternative medium for microbial cultivation, thereby contributing to waste valorization, reducing production costs, and promoting a more sustainable bioprocess without relying on conventional culture media.

Material and Methods

Bacterial Strains and Activation

Five Enterococcus strains (*Enterococcus durans* K48, *Enterococcus faecium* R114, *Enterococcus faecium* T52, *Enterococcus faecium* NB4, and *Enterococcus durans* ND1) previously isolated from traditional Iranian dairy products, were used in this study. Taxonomic identification had been confirmed by molecular assays and 16S rRNA gene sequencing. Stock cultures were maintained in MRS broth (MIRMEDIA, Iran) supplemented with 30% (v/v) glycerol and stored at −80 °C. Prior to experiments, each strain was activated in fresh MRS broth at 37 °C for 24 h to obtain metabolically active cultures (Oleksy‐Sobczak, Klewicka, & Piekarska-Radzik, 2020; Rahnama Vosough, Edalatian Dovom, Habibi Najafi, Javadmanesh, & Mayo, 2022).

Screening of EPS-Producing Strains

The five strains were screened for biomass formation and EPS production. Biomass formation was determined by measuring the dry cell weight (DCW). Following cultivation, the fermentation broth was centrifuged at 4,000 rpm for 10 min. The supernatant was discarded, and the cell pellet was washed twice with distilled water to remove residual medium components. The washed biomass was dried at 80 °C until a constant weight was obtained. Biomass production was expressed as dry cell weight (mg/L) (Choi, Lee, & Paik, 2021). EPS extraction was performed as described by Kavitake et al. (2024) with slight modifications. Briefly, the strains were incubated in MRS broth at 37 °C for 48 h, and the cell biomass was harvested by centrifugation at 4,000 rpm for 10 min. To precipitate proteins, the cell-free supernatant was treated with trichloroacetic acid (TCA) to a final concentration of 10% (w/v) and incubated at 4 °C for 12 h. The precipitated proteins were removed by centrifugation (4,000 rpm, 15 min, 4 °C), and the EPS in the supernatant was subsequently

precipitated by adding three volumes of ice-cold 96% (v/v) ethanol, followed by incubation at 4 °C for 24 h. The EPS precipitate was collected by centrifugation (4,000 rpm, 15 min, 4 °C), dried to a constant weight in a desiccator, and weighed to determine the EPS yield (Hu *et al*., 2021; Kavitake *et al.*, 2024).

Dairy Sludge Source and Characterization

Dairy separator sludge was obtained from a renowned dairy processing plant in Khorasan Razavi, Iran. The raw sludge had an initial pH of 5.5 and a total solids content of 14% (w/v). Chemical characterization was conducted to assess its suitability as a nutrient-rich substrate for microbial cultivation.

Chemical Composition Analysis

Crude protein

Protein content was determined using the Kjeldahl method according to the Iranian National Standard No. 9188-1. Samples were digested with concentrated sulfuric acid in the presence of a catalyst to convert organic nitrogen to ammonium ions. The released ammonia was distilled, absorbed in boric acid, and titrated against standardized hydrochloric acid. A nitrogen-to-protein conversion factor of 6.38 was applied (Iranian National Standardization Organization, 2015).

Fat Content

Fat content was measured using the butyrometric (Gerber) method following Iranian National Standard No. 23626. Samples were mixed with sulfuric acid and amyl alcohol in a butyrometer, centrifuged, and incubated at 65 °C. The fat percentage was determined by reading the height of the fat column on the calibrated scale (Iranian National Standardization Organization, 2024).

Ash Content

The Ash content was determined by incineration. Samples were dried at 105 °C to constant weight and then combusted in a muffle furnace at 550 °C for 4–6 h. After cooling, ash percentage was calculated according to Eq. (1) (Quirino, Palma, Franco, & Detmann, 2023).

$$Ash\ Content = \frac{Weight\ of\ Ash}{Original\ Dry\ Sample\ Weight} \times 100 \quad (1)$$

Total Carbohydrates

Total carbohydrate concentration was determined using the phenol–sulfuric acid method. The samples were reacted with phenol and sulfuric acid, heated in a boiling water bath, cooled, and the absorbance was measured at 490 nm using a UV–Vis spectrophotometer (Unico, Iran). The concentration was calculated against a glucose standard curve (Gao *et al.*, 2022).

Preparation of Dairy Sludge Fermentation Medium

Dairy sludge (pH 5.5, 14% total solids) was used as the basal substrate for bacterial growth. To prepare the dairy sludge as growth medium, two different approaches were employed depending on the experimental purpose. For the RSM experiments, spray-dried dairy sludge was used to ensure substrate homogeneity, facilitate the accurate preparation of defined concentrations, and improve the reproducibility required for statistical optimization. The sludge was pasteurized (72 °C, 30 min) and spray-dried (Buchi, Switzerland) to obtain a powder (Falah, Mortazavi, Danesh, Yazdi, & Ramezani, 2024). The resulting powder was reconstituted in distilled water at 5%, 10%, and 15% (w/v). The medium was supplemented with sucrose, yeast extract, 0.02% (w/v) $MnSO_4$, and 0.2% (w/v) KH_2PO_4, and sterilized at 121 °C for 15 min (Esmaeilnejad-Moghadam, Mokarram, Hejazi, Khiabani, & Keivaninahr, 2019).

In contrast, liquid dairy sludge was used for the scale-up experiment to better simulate industrial processing conditions while avoiding the additional energy consumption and cost associated with spray drying. For scale-up experiments, the pH was adjusted to 6.4 using 1 N HCl. Caseins were removed via heat treatment (95 °C, 15 min) followed by centrifugation and filtration (Che *et al.*, 2025). The clarified supernatant was supplemented with calcium carbonate ($CaCO_3$) to enhance

buffering capacity during fermentation and then sterilized (Fusi, Primicerio, & Monti, 2015).

Growth Assessment of *E. durans* K48

The growth kinetics of the selected strain were evaluated in both MRS broth and the formulated dairy sludge medium supplemented with sucrose and yeast extract. Cultures were incubated at 37 °C for 24 h in a shaker incubator at 250 rpm. To monitor the growth curve, samples were collected at hourly intervals, and the optical density (OD 600) was measured using a UV–Vis spectrophotometer. To account for the inherent turbidity of the dairy sludge medium, samples were appropriately diluted with distilled water prior to spectrophotometric analysis to ensure the absorbance remained within the linear range (Esmaeilnejad-Moghadam *et al.,* 2019).

Optimization of Biomass Production

Response Surface Methodology (RSM) was employed to optimize biomass production using Design-Expert software (version 13). A Central Composite Design (CCD) was employed to evaluate the effects of three independent variables, including (A) dairy sludge concentration, (B) sucrose concentration, and (C) yeast extract concentration. Each variable was evaluated at three distinct levels, as shown in Table 1. Six center-point replicates were included to estimate experimental error. The experimental data were fitted to a second-order polynomial model, expressed as Eq. (2):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^{\,2} + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \quad (2)$$

where Y represents biomass production, β_0 is the intercept, β_i and β_{ii} are linear and quadratic coefficients, respectively, and β_{ij} represents interaction coefficients (Ahmed *et al.,* 2023).

To validate the predictive capability of the developed RSM model, an independent confirmation experiment was performed under the predicted optimal conditions. Three independent experiments were conducted under these optimized conditions, and the mean experimental biomass yield was compared with the value predicted by the model.

Table 1- Levels of independent process variables and their values

Independent variable	Symbol	Unit (w/v)	High Level (+1)	Medium Level (0)	Low Level (−1)
Dairy sludge concentration	A	%	15	10	5
Sucrose concentration	B	%	5	3	1
Yeast extract concentration	C	%	2.5	1.5	0.5

EPS Production, Extraction, and Purification

EPS production was carried out in the optimized dairy sludge medium using a modified procedure adapted from Kavitake et al. (2024). The medium was inoculated with a 3% (v/v) active culture of *E. durans* K48 (adjusted to an optical density of OD_{600}=1.0) and incubated at 37°C for 48 h. Post-incubation, the bacterial cells were harvested by centrifugation at 4,000 rpm for 15 min at 4°C.

To precipitate the proteins, trichloroacetic acid (TCA) was added to the cell-free supernatant to achieve a final concentration of 10% (w/v), and the mixture was incubated at 4°C for 12 h. The precipitated proteins were removed by centrifugation at 4,000 rpm for 20 min at 4 °C. Subsequently, the crude EPS was precipitated from the clarified supernatant by adding three volumes of ice-cold 96% (v/v) ethanol, followed by incubation at 4 °C for 24 h.

The resulting EPS precipitate was collected by centrifugation (4,000 rpm, 20 min, 4°C), dissolved in deionized water, washed twice with ethanol, and dialyzed against deionized water for 48 h at 4°C using a 10 kDa molecular weight cut-off (MWCO) dialysis membrane (with regular water changes every 12 h). Finally, the dialyzed EPS solution was pre-frozen at −80 °C and lyophilized using a freeze-

dryer for 48 h. The purified EPS was weighed to determine the yield and stored at −20 °C for further analysis (Kavitake *et al.*, 2024; Wu, Wu, Pan, Luo, & Zhong, 2024).

Statistical Analysis

Experimental design, model fitting, and response surface plots were conducted using Design-Expert software (version 13.0.1.0). Analysis of variance (ANOVA) was performed to evaluate the statistical significance of the fitted regression model and the linear, quadratic, and interaction effects of the independent variables on biomass production. Statistical significance was considered at $p \leq 0.05$, with highly significant effects indicated at $p \leq 0.001$. Additional statistical analyses were performed using SPSS software (version 27). All experiments were performed in triplicate, and the mean values were used for statistical analysis.

Results and Discussion

Screening of EPS-Producing Strains and Selection of the Superior Strain

Simultaneous assessment of biomass formation and EPS production enabled clear discrimination among the screened Enterococcus strains. Biomass measurements revealed statistically significant variation ($p \leq 0.001$), allowing classification of the strains into distinct homogeneous groups. Among the isolates, strain K48 exhibited the highest biomass concentration (approximately 2.1 g/L), followed by R114 (1.7 g/L), NB4 (1.5 g/L), ND1 (0.9 g/L), and T52 (0.7 g/L). The superior growth of K48 indicates a greater physiological tolerance to the fermentation environment and implies more efficient substrate uptake and energy metabolism, which are commonly associated with enhanced polysaccharide biosynthesis (Netrusov *et al.*, 2023). As illustrated in Table 2, EPS production followed a growth-associated pattern, consistent with the biomass trends. EPS yields among the tested strains ranged from 0.12 to 0.40 g/L , with K48 achieving the maximum titer of 0.40 g/L (denoted by the letter ‘a’ in Table 2). This elevated productivity may be attributed to inherent genetic variations in the regulation of glycosyltransferases and precursor-sugar biosynthetic pathways (Cui, Jiang, Hao, Qu, & Hu, 2017). Similar EPS production levels (approximately 0.40–0.50 g/L) have been reported for Enterococcus strains cultivated in carbohydrate-rich media (Kowsalya *et al.*, 2023), supporting the reliability of the present results. The lower performance of strains such as T52 and ND1 may be attributed to reduced expression of EPS operons or less efficient carbon-source metabolism. Such strain-specific metabolic limitations have been previously reported in LAB, where energy allocation between growth and exopolysaccharide formation varies among species and isolates (Liang, Wang, Li, & Liu, 2024). From a bioprocessing perspective, selecting a strain capable of achieving both high biomass and EPS productivity is crucial for cost-effective fermentation, particularly when low-value substrates such as dairy sludge are used. The markedly superior performance of *E. durans* K48 supports its selection as the most promising candidate for subsequent optimization studies and scale-up development.

Table 2- Comparison of biomass concentration and EPS production among the screened Enterococcus strains (R114, T52, K48, ND1, and NB4)

Bacterial strains	EPS production (g/L)	Biomass content
R114	0.226 ± 0.028^{c}	1.702 ± 0.050^{b}
T52	0.112 ± 0.006^{e}	0.773 ± 0.018^{e}
K48	0.401 ± 0.017^{a}	2.108 ± 0.016^{a}
ND1	0.158 ± 0.021^{d}	0.978 ± 0.010^{d}
NB4	0.298 ± 0.005^{b}	1.501 ± 0.004^{c}

Different letters above columns with the same color denote statistically significant differences among strains ($p < 0.05$).

Chemical Properties of Dairy Sludge

The chemical profile of dairy sludge—comprising protein, ash, fat, and total carbohydrates—is presented in Table 3. The sludge contained 29.32±0.42%protein (dry basis), indicating a substantial concentration of nitrogenous compounds derived from residual milk proteins such as casein and whey (Prazeres, Carvalho, & Rivas, 2012). The ash content was 9.56±0.73%, reflecting a rich mineral profile (including calcium, phosphorus, and magnesium) essential for supporting microbial metabolism (Kwapinska, Pisano, & Leahy, 2023).

Notably, the fat content reached 32.73±0.52%, likely due to incomplete fat separation during industrial processing and the subsequent accumulation of fat globules in the sludge (Ganta, Bashir, & Das, 2022). Regarding the carbon source, total carbohydrate content exhibited a slight but statistically significant decrease ($p<0.05$) from 30.17±0.93% in the raw sludge to 28.29±0.50% after spray-drying. This reduction is potentially attributable to the partial thermal degradation or Maillard reactions involving lactose and other reducing sugars during the drying process (Ziaee, O'Connor, Murphy, & O'Reilly, 2023). Nevertheless, the high residual carbohydrate levels confirm the suitability of dried dairy sludge as a valuable carbon source for Enterococcus cultivation.

These results are generally consistent with existing literature, although some variations exist. For instance, McCarthy (2011) reported that dairy sludge (dry basis) typically contains 40–53% nitrogenous compounds and approximately 31.3% lactose, but significantly lower fat levels (1.7–2.3%). The observed discrepancies in fat and protein concentrations compared to previous studies may be attributed to differences in milk origin, specific dairy processing techniques (e.g., cheese vs. yogurt production), and seasonal variations in sludge sampling.

Table 3- Chemical composition of the dairy sludge

Sample	Protein (%)	Ash (%)	Fat (%)	Total carbohydrate (%) before spray drying	Total carbohydrate (%) after spray drying
Dairy sludge	29.32 ± 0.42	9.56 ± 0.73	32.7 ± 0.52	30.17 ± 0.93	28.50 ± 0.50

Comparative Growth of *E. durans* K48 in Dairy Sludge and MRS Media

The growth profiles of *E. durans* K48 cultivated in MRS broth and dairy sludge medium over a 24 h fermentation period are shown in Fig. 1. Both media supported a characteristic sigmoidal growth pattern; however, distinct differences were observed in their kinetic parameters.

A prolonged lag phase was observed in the dairy sludge medium, with exponential growth initiated after approximately 6–7 h. In contrast, cells grown in MRS broth adapted more rapidly, entering the exponential phase by hour 3. This initial delay in dairy sludge can be attributed to the time required for the bacterial cells to synthesize extracellular enzymes needed to metabolize complex substrates (such as residual casein and lactose) compared to the readily bioavailable nutrients in MRS (Esmaeilnejad-Moghadam *et al.*, 2019).

Interestingly, following the adaptation period, *E. durans* K48 exhibited a rapid acceleration in cell density within the dairy sludge medium (between 8 and 14 h), reaching a plateau (OD_600≈1.60-1.65) by hour 14. Conversely, growth in MRS broth progressed at a steady rate, attaining a higher maximum cell density (OD_600≈1.90) at hour 24. The earlier onset of the stationary phase in dairy sludge may result from the depletion of specific micronutrients or the accumulation of organic

acids during fermentation. Nevertheless, achieving nearly 85% of the total biomass produced in refined MRS broth confirms that dairy sludge is a highly effective, low-cost nutrient substrate for *E. durans* cultivation and subsequent EPS production.

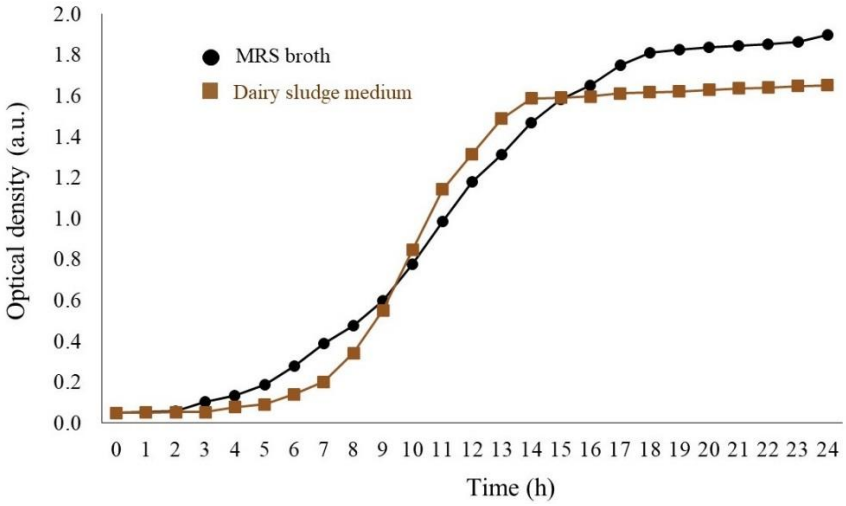

Fig. 1. Comparative growth curves of E. durans K48 in MRS broth and dairy sludge medium during 24 h of incubation

Optimization of Biomass Production Using Response Surface Methodology

A central composite design (CCD) comprising 21 experimental runs was applied to evaluate the effects of (A) dairy sludge concentration, (B) sucrose concentration, and (C) yeast extract concentration, on biomass production by *E. durans* K48. The experimental design matrix, including the actual concentrations of the independent variables and the corresponding experimental and predicted biomass yields for all 21 runs, is presented in Table 4. The experimental responses were fitted to a second-order polynomial model (Eq. 3), which is commonly used to describe nonlinear interactions among process variables:

$$Y = 7713.43 - 18.99A + 53.58B + 504.95C + 49.63AB + 72.70AC + 74.99BC + 396.43A^2 + 22.71B^2 - 391.87C^2 \quad (3)$$

Analysis of variance (ANOVA) indicated that the regression model was highly significant ($F = 39.61$, $p < 0.0001$), demonstrating a strong relationship between the tested variables and biomass production. ANOVA showed that the model was highly significant, and several linear, interaction, and quadratic terms significantly affected biomass production ($p \leq 0.0059$), indicating that variations in dairy sludge, sucrose, and yeast extract concentrations markedly influenced cell growth. The model exhibited a high coefficient of determination ($R^2 = 0.9701$), with an adjusted R^2 of 0.9456, indicating a good fit between the model and the experimental data. The coefficient of variation (CV) was low (1.48%), suggesting good precision and reliability of the experimental results. The predicted R^2 was 0.3142, while the adequate precision was 21.1384, exceeding the desirable value of 4 and indicating an adequate signal-to-noise ratio for the model. The positive coefficients for sucrose and yeast extract indicate that biomass formation was enhanced by increasing the availability of easily metabolizable carbon and nitrogen sources. Although the interaction coefficients (AB, AC, and BC) were positive, their corresponding effects were not statistically significant ($p > 0.05$); therefore, no significant synergistic interaction among the tested variables could be established. The lack-of-fit test was not significant ($p = 0.0864$), confirming that the

developed model adequately represented the experimental data within the studied range.

Table 4- Central composite design matrix for biomass production by *E. durans* K48, including actual and predicted values

Run	A: Dairy sludge concentration (%)	B: Sucrose concentration (%)	C: Yeast extract concentration (%)	Bio Mass (mg/L)	Predicted Value (mg/L)
1	10	3	1.5	7590	7713.43
2	10	3	1.5	7660	7713.43
3	5	5	2.5	8290	8270.87
4	15	1	2.5	8100	8121.17
5	10	3	1.5	7580	7713.43
6	15	5	2.5	8440	8477.57
7	15	1	0.5	7150	7115.83
8	5	5	0.5	7240	7256.40
9	15	5	0.5	7110	7172.28
10	10	3	1.5	7780	7713.43
11	5	3	1.5	8230	8128.85
12	10	3	2.5	7970	7826.51
13	10	3	1.5	7610	7713.43
14	15	5	0.5	7210	7172.28
15	5	1	2.5	8100	8113.01
16	10	3	0.5	6730	6816.60
17	10	5	1.5	7940	7789.72
18	10	3	0.5	6910	6816.60
19	5	5	2.5	8180	8270.87
20	15	3	1.5	8140	8090.87
21	10	3	1.5	7760	7713.43

Main and Interactive Effects of Process Variables on Biomass Yield

Fig. 2 presents the effects of dairy sludge concentration (A), sucrose concentration (B), and yeast extract concentration (C) on biomass production by *E. durans K48*, as illustrated by three-dimensional response surface plots. The plots reveal that biomass production was influenced not only by the individual levels of the variables but also by their combined effects.

As shown in Fig. 2a, the combined effect of dairy sludge and yeast extract markedly influenced biomass formation. Increasing yeast extract concentration enhanced biomass production up to an optimum level, confirming the critical role of nitrogen availability in supporting bacterial growth. However, further increases in yeast extract resulted in a slight decline in the response, which is consistent with the negative quadratic coefficient of C^2 in the fitted model and suggests the presence of an optimum concentration range.

Fig. 2b illustrates the interaction between dairy sludge and sucrose concentrations. Biomass production increased when both variables were maintained at moderate-to-high levels, indicating a favorable combined effect between the complex nutrients supplied by dairy sludge and the readily metabolizable carbon source provided by sucrose (Ashekuzzaman, Forrestal, Richards, & Fenton, 2019; Kwapinska *et al.*, 2023). However, increasing sucrose concentration beyond the optimal range did not result in a substantial additional improvement in biomass yield.

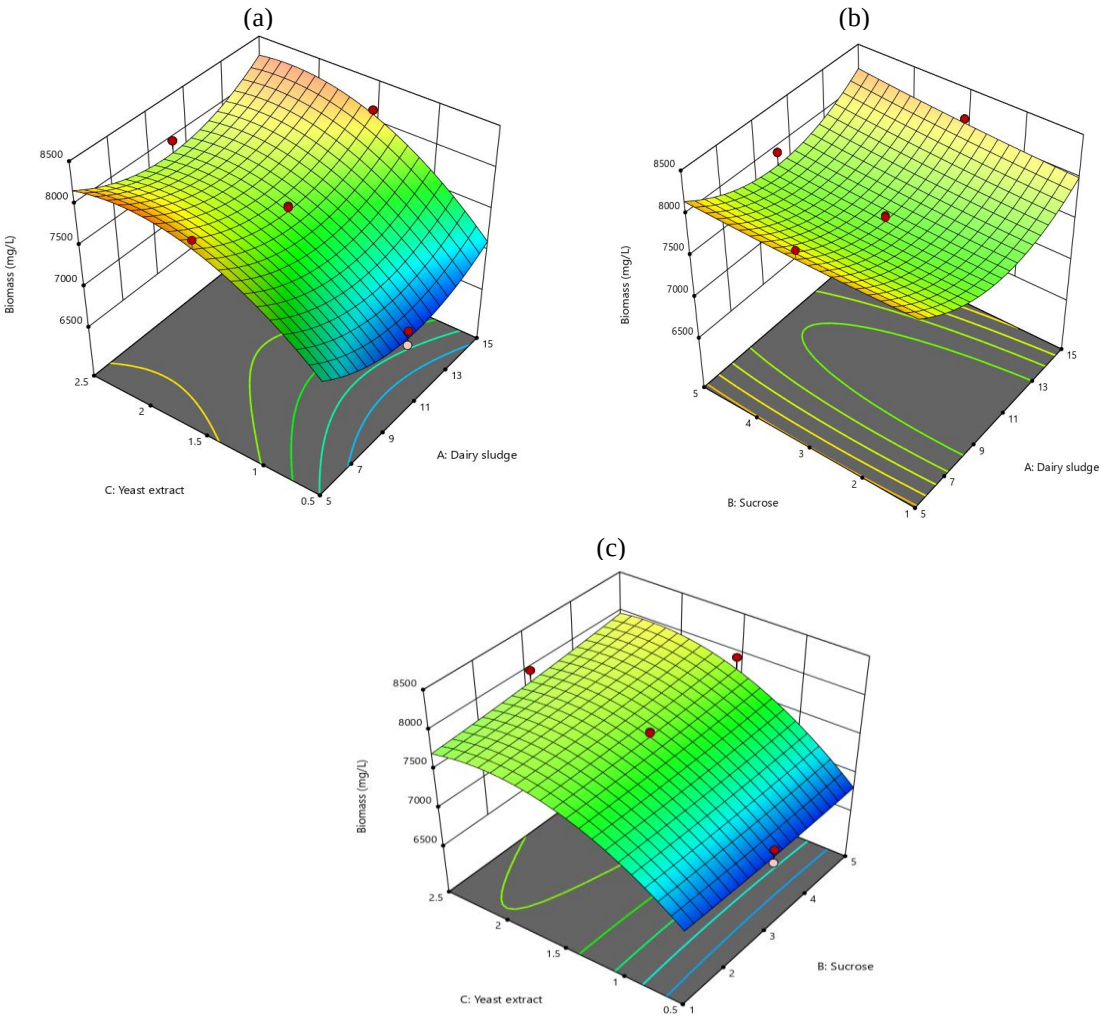

Fig. 2. Three-dimensional response surface plots showing the interactive effects of (a) dairy sludge concentration and yeast extract concentration, (b) dairy sludge concentration and sucrose concentration, and (c) sucrose concentration and yeast extract concentration on biomass production by *E. durans* K48

As presented in Fig. 2c, biomass production was maximized when sucrose and yeast extract concentrations were properly balanced, emphasizing the importance of coordinated carbon and nitrogen supplementation. Deviations toward either insufficient or excessive levels of these components adversely affected cell growth (Esmaeilnejad-Moghadam *et al.*, 2019). Overall, the curved response surfaces and the elliptical nature of the contour plots suggest the presence of interaction effects among the investigated variables. These findings indicate that biomass production is governed by the combined influence of medium components, and therefore, optimization should be performed simultaneously rather than by varying one factor at a time.

The stimulatory effect of dairy sludge on biomass production can be attributed to its complex nutritional composition. Dairy sludge contains residual lactose, proteins, peptides, lipids, and minerals derived from dairy processing effluents (McCarthy, 2011). These constituents may serve as carbon and nitrogen

sources as well as growth-promoting factors for lactic acid bacteria. Fermentable carbohydrates such as lactose provide energy for metabolism, whereas proteins and peptides contribute amino acids required for biosynthesis. In addition, minerals and trace elements may enhance enzymatic activity and overall metabolic efficiency. Therefore, incorporation of dairy sludge into the culture medium creates a nutritionally enriched environment that supports microbial proliferation. Similar observations have been reported for other dairy by-products, such as whey, which have been successfully used as low-cost medium for microbial biomass production (Esmaeilnejad-Moghadam *et al.*, 2019), Collectively, these results highlight the potential of dairy sludge as an economical medium and support its valorization as a sustainable resource in microbial bioprocessing.

Determination of Optimal Conditions and Model Validation

Biomass yield varied from 6730 to 8440 mg/L across the CCD experiments. The maximum biomass concentration (8440 mg/L) was obtained at A = 15%, B = 5%, and C = 2.5%. This combination likely provided a favorable balance of carbon, nitrogen, and complex nutrients from dairy sludge, and create favorable conditions for efficient cell proliferation. To verify the accuracy of the developed quadratic model, predicted values were compared with experimental data. The scatter plot presented in Fig. 3 showes that most data points were closely distributed along the line of equality, indicating a strong agreement between the model-predicted and experimentally observed biomass yields. The absence of major deviations or outliers further confirms the robustness, reliability, and predictive capability of the RSM model for optimizing biomass production in dairy-sludge-based media.

The predicted optimal conditions were experimentally validated through three independent confirmation experiments. The experimental results were in close agreement with the model predictions, confirming the predictive capability and reliability of the developed RSM model.

Exopolysaccharide Production, Extraction, and Purification

Following optimization of the fermentation parameters, EPS production was evaluated under the identified optimal conditions (dairy sludge 15%, sucrose 5%, and yeast extract 2.5%). Under these conditions, *E. durans* K48 yielded approximately 394.2 mg/L of EPS. After extraction and lyophilization, the recovered EPS appeared as a fine white powder with a fibrous, cotton-like texture, which is typical characteristic of highly polymerized microbial polysaccharides. The EPS yield obtained in this study was comparable to several previously reported values. For instance, Jia *et al.* (2019) reported an EPS production of 130 mg/L using *E. faecium* WEFA23, while Zanzan *et al.* (2023) achieved 382.27 mg/L by *E.mundtii* A2, under their optimized fermentation conditions.

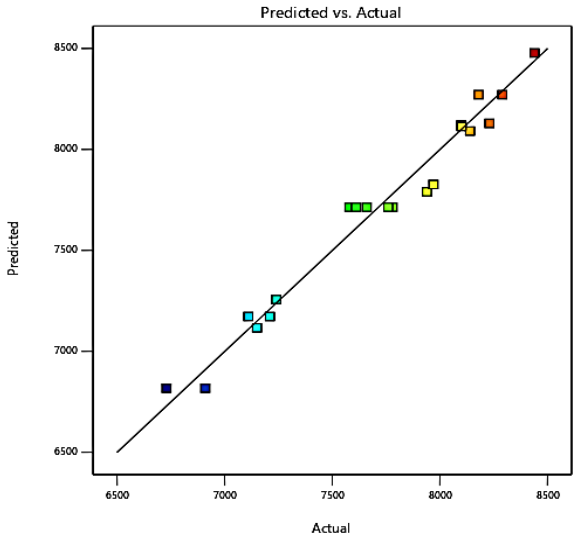

Fig. 3. Comparison between predicted and experimental values of biomass production based on the developed regression model

The relatively high yield observed in the present work may be attributed to the combined effect of the selected strain and the optimized nutritional composition of the culture medium determined, which was established using response surface methodology. These findings indicate that *E. durans* K48 is a promising candidate for efficient EPS production. Furthermore, the use of dairy sludge as a fermentation substrate highlights the potential of this process as a cost-effective and sustainable approach for microbial polysaccharide production with possible industrial applications.

While the obtained EPS yield and the use of dairy sludge as a low-cost substrate are promising, further investigations are required to fully assess the potential of the produced EPS and the strain itself for future applications. Comprehensive structural and functional characterization of the EPS, including analyses of monosaccharide composition, molecular weight distribution, rheological behavior, and other physicochemical and functional properties, was beyond the scope of the present study and is being addressed in a separate follow-up investigation focused on EPS characterization. In parallel, the potential application of the strain itself requires further consideration. The primary objective of the present study was to evaluate the feasibility of dairy sludge as a sustainable and low-cost fermentation substrate for microbial biomass and EPS production, rather than to assess the suitability of *E. durans* K48 for direct food or probiotic applications. Therefore, further safety assessments, including genomic analysis for the detection of potential virulence factors and antibiotic resistance determinants, are needed before considering this strain for such applications (Farkhondeh, Yavarmanesh, Tabatabaei Yazdi, & Edalatiane, 2023; Zanzan *et al.*, 2023).

Conclusion

This study demonstrated the feasibility of utilizing dairy sludge as a sustainable, nutrient-rich medium for microbial biomass and exopolysaccharide (EPS) production. Among the screened isolates, *E. durans* K48 exhibited the highest growth performance and EPS-producing capacity in the dairy-sludge-based medium. Response surface methodology (RSM) identified the optimal cultivation conditions as 15% dairy sludge, 5% sucrose, and 2.5% yeast extract, under which the maximum biomass and EPS yields reached 8440 mg/L and 394.2 mg/L, respectively. These results highlight dairy sludge as a valuable, low-cost resource capable of supporting

efficient microbial metabolism, thereby demonstrating the potential of this optimized bioprocess for the cost-effective valorization of dairy industry waste. The robust performance of *E. durans* K48 further underscores its applicability in the food, pharmaceutical, and biotechnology-related sectors. To facilitate industrial implementation, future research should focus on process scale-up, detailed structural and functional characterization of the recovered EPS, and a comprehensive techno-economic assessment of the integrated bioprocess.

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Author Contributions

Nazanin Taheri Nasab: Conceptualization, Methodology, Software, Investigation, Formal analysis, Data Curation, Writing–Original Draft, Visualization. **Mohammad Reza Edalatian Dovom**: Conceptualization, Resources, Supervision, Project administration, Funding acquisition, Writing–Review & Editing. **Marzieh Moeenfard**: Conceptualization, Validation, Supervision, Writing–Review & Editing. **Leila Rouzbeh Nasiraie**: Conceptualization, Validation, Supervision, Writing–Review & Editing.

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ارزش‌آفرینی از لجن لبنی به‌عنوان بستری کم‌هزینه برای افزایش تولید زیست‌توده و اگزوپلی‌ساکارید توسط *انتروکوکوس دورانس* K48

نازنین طاهری نسب[۱]- محمد رضا عدالتیان دوم[۱*]- مرضیه معین فرد[۱]- لیلا روزبه نصیرایی[۲]

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چکیده

پساب صنایع لبنی، به‌ویژه لجن لبنی غنی از مواد آلی، یک منبع امیدبخش و پایدار برای کاربردهای بیوتکنولوژیک به شمار می‌رود. این پژوهش، پتانسیل لجن لبنی را به‌عنوان یک محیط‌کشت مقرون به صرفه برای تولید همزمان زیست‌توده و اگزوپلی ساکاریدها (EPS) توسط سویه‌های منتخب انتروکوکوس ارزیابی کرد. ابتدا پارامترهای فیزیکوشیمیایی لجن لبنی مشخص شد که ترکیبی از ۳۲/۷۰٪ چربی، ۲۹/۳۲٪ پروتئین، ۹/۵۶٪ خاکستر و ۳۰/۱۷ ٪ کربوهیدرات (که پس از فرآیند خشک کردن به ۲۸/۵۰٪ تنظیم شد) را نشان داد. پنج سویه از طریق یک رویکرد کمی وابسته به کشت غربالگری شدند تا رشد و تولید EPS آنها ارزیابی شود. در میان جدایه‌های آزمایش شده، *انتروکوکوس دورانس* K48 بالاترین پتانسیل تولید را نشان داد و برای مطالعه بیشتر انتخاب شد. از روش سطح پاسخ (RSM) برای بهینه‌سازی غلظت اجزای کلیدی محیط‌کشت: لجن لبنی، ساکارز و عصاره مخمر استفاده شد. تحت شرایط بهینه (۱۵٪ لجن لبنی، ۵٪ ساکارز و ۲/۵٪ عصاره مخمر)، حداکثر بازده زیست‌توده برابر با ۸۴۴۰ میلی‌گرم بر لیتر و بازده EPS برابر با ۳۹۴/۲ میلی‌گرم بر لیتر به‌دست آمد. این یافته‌ها نشان می‌دهند که لجن لبنی می‌تواند به‌عنوان بستری مناسب و پایدار برای کشت انتروکوکوس مورد استفاده قرار گیرد و ضمن فراهم کردن امکان تولید کارآمد زیست‌توده و EPS، به ارزش‌آفرینی از پسماندهای حاصل از فرآوری صنایع لبنی نیز کمک کند.

واژه‌های کلیدی: ارزش‌آفرینی، باکتری‌های اسیدلاکتیک، بهینه‌سازی محیط‌کشت، زیست توده، لجن لبنی

۱- گروه علوم و مهندسی صنایع غذایی، دانشکده کشاورزی، دانشگاه فردوسی مشهد، مشهد، ایران
(*- نویسنده مسدول : Email: edalatian@um.ac.ir)
۲- گروه علوم و صنایع غذایی، دانشگاه آزاد اسلامی واحد نور، نور، ایران

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