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# OPTIMIZATION OF METAL ION CONCENTRATION IN YEAST EXTRACT-PEPTONE MEDIUM FOR ENHANCED BIOETHANOL FERMENTATION USING RESPONSE SURFACE METHODOLOGY

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## ABSTRACT

**Bioethanol is a renewable eco-friendly alternative energy source produced by fermenting simple sugars using *Saccharomyces cerevisiae*. However, stress factors during fermentation can reduce yeast efficiency. Optimizing the metal ion supplementation in the growth medium is one strategy to mitigate these effects and improve ethanol yield. The present study was aimed to determine the optimal concentrations of calcium, magnesium, and zinc ions for maximizing ethanol production. Fermentation was carried out in yeast extract-peptone (YEP) medium supplemented with these metal ions. Optimization was conducted using response surface methodology with a central composite design (RSM-CCD). The experimental steps included yeast cell rejuvenation, media preparation, starter culture development, and fermentation. Optimal concentrations of calcium, magnesium, and zinc were 26.36, 368.18, and 66.82 mg $L^{-1}$, respectively. Under these conditions, the predicted ethanol yield was 0.567 g $g^{-1}$, while the validation experiment produced 0.274 ± 0.018 g $g^{-1}$. This represents a 20.7% increase compared as to the center point (0.274 vs 0.227 g $g^{-1}$). Although optimization enhanced ethanol yield, further refinement of fermentation conditions and medium composition is needed to reduce the gap between predicted and experimental values and to improve overall fermentation performance.**

**Keywords:** Bioethanol, fermentation, metal ions supplementation, response surface method, *Saccharomyces cerevisiae*

## INTRODUCTION

The world's energy consumption continues to rise, with International Energy Agency (2023) projecting that global oil demand from petrochemicals is likely to rise from 2022 to 2028, reaching 105.7 million barrels $day^{-1}$. However, fossil fuel availability is limited, raising concerns about economic and energy security if global dependence on these non-renewable resources persists (Wang and Azam, 2024). The long-term sustainability of fossil fuels is also uncertain, as the imbalance between production and consumption drives price volatility (Martins *et al.*, 2019). Further, fossil fuels contribute significantly to the environmental damage through incomplete combustion, which enhances $CO_2$ emissions - reaching the levels as high as 403 ppm (Energy Information Administration, 2017). These emissions exacerbate climate change, affecting water, energy, and food security while intensifying rainwater evaporation, hydrological cycles, heat waves, and tropical storms. They also contribute to the increased flooding and prolonged dry seasons (Azni *et al.*, 2023). In response to these

adverse impacts, there is a growing interest in exploring the renewable and sustainable energy alternatives. Renewable energy sources are growing at an average rate of 16% per year over past decade (Santos *et al.*, 2023). Among these, bioethanol stands out as a promising alternative due to its recyclability, clean combustion, and potential to reduce greenhouse gas emissions by 90% as compared to the fossil fuels (Sydney *et al.*, 2019). In 2021, global bioethanol production reached 27 billion gallons, with USA leading at 15 billion gallons, while Indonesia contributed 5.1% of the total 14.4% ethanol production in the Asia-Pacific region (British Petroleum, 2019; Fernandes *et al.*, 2022).

Bioethanol is produced by converting simple sugars into ethanol through alcoholic fermentation using microorganisms, particularly the yeast *Saccharomyces cerevisiae*. This yeast is widely used due to its high ethanol tolerance, and efficient glucose-to-ethanol conversion (Tse *et al.*, 2021). However, bioethanol is costly as compared to the fossil fuels due to the limitations in production process, such as yeast cell exposure to environmental stresses, including high ethanol concentrations, temperature fluctuations, and nutrient limitations, which reduce ethanol yields (Eardley and Timson, 2020). These challenges can be addressed through strategies like C and N sources optimization, metal ions supplementation and use of ethanol-tolerant yeast strains (Ahmed *et al.*, 2020; Jiang *et al.*, 2023).

In bioethanol production nutrition is required in the fermentation media. Feedstock may contain simple sugars like glucose or complex polysaccharides such as starch or cellulose. Complex feedstock require pretreatment to release fermentable sugars (Saggi and Dey, 2019). Besides, C sources, N is essential for yeast growth, serving as a building block for amino acids, nitrogenous bases and other metabolites (Gobert *et al.*, 2019). Nitrogen can be provided through organic sources (like yeast extract peptone (YEP)] or inorganic sources [like yeast nitrogen base (YNB)]. YEP is often preferred because it contains bioavailable N derived from yeast extract and bacteriological peptone. Ishmayana *et al.* (2012) demonstrated higher ethanol yields, lower residual glucose levels, and faster cell growth when YEP was used instead of YNB. Therefore, YEP was selected in this study to optimize fermentation.

Metal ions are essential micronutrients in fermentation media, playing critical roles in cellular functions and metabolic processes. Potassium contributes to osmoregulation and enzyme activation, magnesium serves as a cofactor for key glycolytic enzymes, manganese supports biomass production and amino acid metabolism, iron acts as a metabolic cofactor, calcium facilitates yeast flocculation and contributes to cell wall conformation, and zinc activates alcohol dehydrogenase and supports vital metabolic enzymes (Ismail *et al.*, 2014; Barros de Souza *et al.*, 2016; Kolakowski *et al.*, 2020; Ribeiro-Filho *et al.*, 2021). Xue *et al.* (2008) demonstrated that magnesium, calcium, and zinc significantly enhanced ethanol tolerance in yeast, while manganese, iron, and cobalt have less pronounced effects.

Wang *et al.* (2024) demonstrated that supplementing the fermentation medium with 361.54 mg $L^{-1}$ magnesium ions resulted in a 5.5-fold increase in ethanol content as compared to the fermentation without magnesium addition. Also, when combined with other nutrients, magnesium ions further improved ethanol yield by 1.1-fold. Hargono *et al.* (2023) reported positive effect of calcium on bioethanol production than ferrous ions. While Kosiv (2024) found that both calcium and zinc enhanced the fermentation rate, with calcium showing a significantly higher impact (up to 21.9% increase) as compared to zinc. While these studies examined the effects of individual metal ions, none studied their combined impact. Recent work by our group revealed that the optimum combination of calcium, magnesium, and zinc ions in YNB medium resulted in 4.8% increase in ethanol yield as compared to the center point of the experiment (Ishmayana *et al.*, 2025). The present study was aimed to evaluate the optimum concentrations of calcium, magnesium, and zinc in YEP medium to enhance ethanol yield by using response surface methodology. Response surface methodology-Central composite design (RSM-CCD) is widely used to achieve optimal fermentation conditions. This statistical technique allows researchers to model and optimize multiple parameters simultaneously, reducing the number of experimental runs while improving the accuracy and efficiency (Yolmeh *et al.*, 2014). RSM-CCD has successfully been applied in fields such as for citric acid production (Książek *et al.*, 2023), biodiesel production (Asaad *et al.*, 2024), lactic acid production (Chaisu *et al.*, 2014), drug research (Akhtar *et al.*, 2024), and bioethanol production (Alalyani *et al.*, 2023).

## MATERIALS AND METHODS

### *Materials*

*S. cerevisiae* Pinnacle S yeast culture was a generous gift received from Anthony Heinrich from AB Mauri, Australia. The chemicals used for growth culture medium were purchased from HiMedia, and the chemicals used for analysis were purchased from Sigma Aldrich. All the chemicals used were of analytical grade unless otherwise stated.

### *Yeast strain and maintenance*

*S. cerevisiae* Pinnacle S yeast was cultured on YEP agar slants containing (w/v): 0.5% yeast extract, 0.5% bacteriological peptone, 0.3% ammonium sulphate, 0.3% monopotassium phosphate, 1% glucose, and 1.5% agar. The medium had an approximate pH of 7.0. The agar slants were stored at 4°C and sub-cultured after every 6 months to maintain viability.

### *Growth media and starter culture conditions*

Starter cultures were prepared by transferring 1-2 loops of yeast colonies from the agar slant into an inoculum broth medium. The yeast was cultured in YEP medium (pH, ~ 7) for ~16 h at room temperature with constant shaking at 180 rpm. To ensure sufficient dissolved oxygen, the ratio of Erlenmeyer flask size to culture volume was maintained at 4:1. The resulting starter culture was then used to inoculate the fermentation medium to achieve a final concentration of ~$10^6$ living cells $mL^{-1}$.

### *Preliminary experiment and sampling condition*

The experiments were conducted in YEP medium containing (w/v) 0.5% yeast extract, 0.5% bacteriological peptone, 0.3% ammonium sulphate, 0.3% monopotassium phosphate, and 20% glucose (pH, ~ 7). The initial concentrations of calcium, magnesium, and zinc in the medium were determined using atomic absorption spectrophotometry (AAS) [Shimadzu AA-7000]. For the initial fermentation experiment, 50 mL medium were inoculated with starter culture, and fermentation was carried out in an orbital shaker incubator at 180 rpm and 30°C for 96 h. Samples were drawn every 6 h for the first 24 h, and subsequently every 12 h. The optical density, cell viability, glucose concentration, and ethanol concentration of the samples were measured.

### *Growth curve*

Yeast growth was determined by recording the optical density at λ = 600 nm ($OD_{600}$) using a spectrophotometer (Thermo Scientific™ GENESYS™ 10S UV-Vis). Samples were diluted with distilled water when required.

### *Determination of cell viability, glucose and ethanol*

Cell viability was determined as described by Smart *et al.* (1999) by differentiating living and dead cells using methylene violet. Glucose concentration was measured using the method proposed by Walker and Harmon (1996), based on the alkaline ferricyanide method. Ethanol concentration was determined using an alcohol dehydrogenase assay as described by Ishmayana *et al.* (2015a).

### *Experimental design and statistical analysis*

The experiments were designed using response surface methodology (RSM) with a central composite design (CCD) in Minitab 20 statistical software. The low and high levels of each factor assessed were as under:

| Factors | Levels | | | | |
|---|---|---|---|---|---|
| | -α | -1 | 0 | +1 | +α |
| $Ca^{2+}$ concentration (mg $L^{-1}$) | 26.36 | 40 | 60 | 80 | 93.64 |
| $Mg^{2+}$ concentration (mg $L^{-1}$) | 31.82 | 100 | 200 | 300 | 368.18 |
| $Zn^{2+}$ concentration (mg $L^{-1}$) | 33.18 | 40 | 50 | 60 | 66.82 |

The experimental runs followed in RSM-CCD for optimization of ethanol fermentation were as under:

| Run order | $Ca^{2+}$ (mg $L^{-1}$) | $Mg^{2+}$ (mg $L^{-1}$) | $Zn^{2+}$ (mg $L^{-1}$) |
|---|---|---|---|
| 1 | 60.00 | 200.00 | 66.82 |
| 2 | 60.00 | 200.00 | 50.00 |
| 3 | 60.00 | 200.00 | 50.00 |
| 4 | 80.00 | 300.00 | 40.00 |
| 5 | 60.00 | 368.18 | 50.00 |
| 6 | 60.00 | 200.00 | 50.00 |
| 7 | 80.00 | 100.00 | 60.00 |
| 8 | 60.00 | 31.82 | 50.00 |
| 9 | 40.00 | 300.00 | 40.00 |
| 10 | 40.00 | 300.00 | 60.00 |
| 11 | 60.00 | 200.00 | 33.18 |
| 12 | 60.00 | 200.00 | 50.00 |
| 13 | 60.00 | 200.00 | 50.00 |
| 14 | 40.00 | 100.00 | 60.00 |
| 15 | 80.00 | 300.00 | 60.00 |
| 16 | 60.00 | 200.00 | 50.00 |
| 17 | 93.64 | 200.00 | 50.00 |
| 18 | 26.36 | 200.00 | 50.00 |
| 19 | 80.00 | 100.00 | 40.00 |
| 20 | 40.00 | 100.00 | 40.00 |

The ethanol yield were estimated as per the method described by us previously (Ishmayana *et al.*, 2025). A factor was considered to have a significant effect on the response when the p value < 0.05.

***Experiment and sampling conditions***

The experiments were conducted in YEP medium containing 0.5% yeast extract, 0.5% bacteriological peptone, 0.3% ammonium sulphate, 0.3% mono-potassium phosphate and 20% glucose. The optimization of calcium, magnesium, and zinc ion content was designed using RSM with CCD. Fermentation was performed in a B-One SIC-50 orbital shaking incubator at 30°C for 72 h, after which the glucose and ethanol contents were determined.

***Validation of optimum conditions for metal ion supplementation on bioethanol production***

Validation of the optimum fermentation conditions was performed using the parameters obtained from RSM-CCD. This step was aimed to assess the effect of calcium magnesium, and zinc ion supplementation on ethanol yield. The validation experiments were conducted in triplicate to ensure reproducibility.

## RESULTS AND DISCUSSION

***Preliminary experiment***

A preliminary experiment was conducted to determine the concentrations of calcium, magnesium, and zinc ions in YEP medium. The results from AAS (Table 1) were subsequently used to calculate the volume of metal ion stock solution required for supplementation into the fermentation medium. The $OD_{600}$ analysis results from basal fermentation of YEP medium are given in Fig. 1. The LSD Fisher post-hoc test indicates that the $OD_{600}$ values at 72 and 84 h fall within the same group, suggesting non-significant difference between these time points. This finding aligns with Olivares-Marin *et al.* (2018) who reported that *S. cerevisiae* reached a stationary $OD_{600}$ value around 24h when 10% glucose was used in fermentation medium, maintaining this value up to 48 h. However, in this study, a higher initial glucose concentration (20%, w/v) was used, resulting in a delayed stationary phase observed at 72 h.

**Table 1: Metal concentrations in YEP medium determined by AAS**

| Metal ion | Concentration (mg $L^{-1}$) |
|---|---|
| $Ca^{2+}$ | 3.39 ± 0.04 |
| $Mg^{2+}$ | 3.09 ± 0.61 |
| $Zn^{2+}$ | 27.10 ± 0.31 |

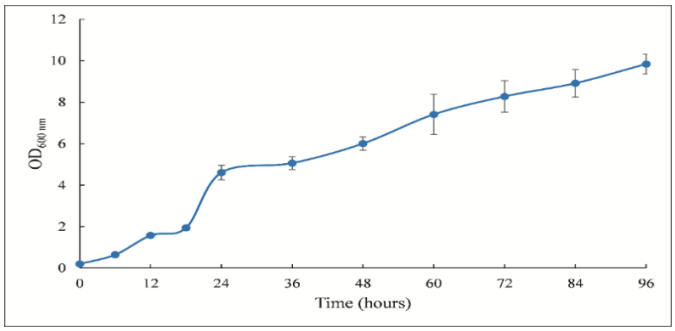


**Fig. 1: Interval plot of $OD_{600}$ during 96-h fermentation in YEP medium with 20% glucose. Data represent the mean of triplicate experiments; the error bars indicate standard deviations**

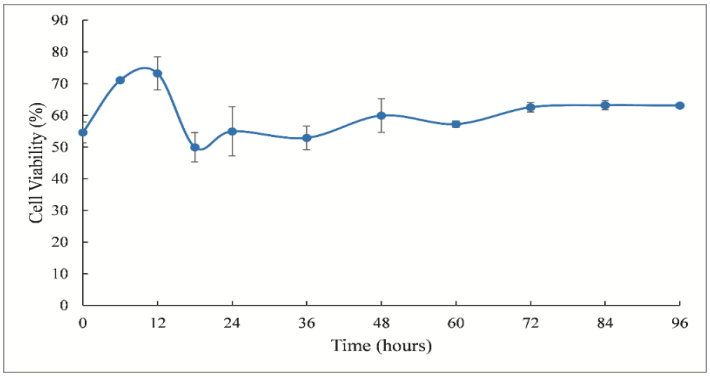


**Fig. 2: Interval plot of cell viability during 96-h fermentation in YEP medium with 20% (w/v) glucose. Data represent the mean of triplicate experiments; the error bars indicate standard deviations**

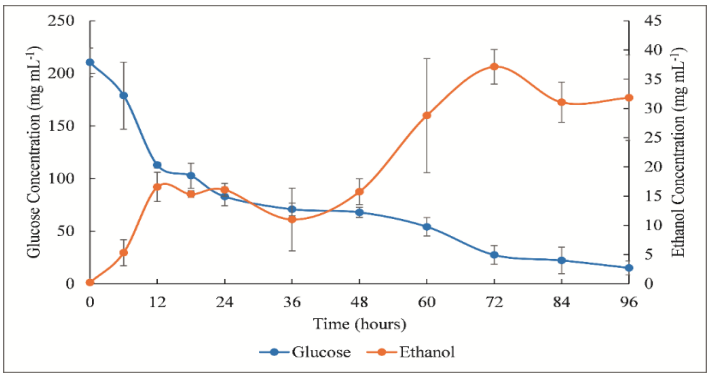


**Fig. 3: Interval plot of glucose and ethanol concentration during a 96-hour fermentation in YEP medium with 20% w $v^{-1}$ glucose. Data represent the mean of triplicate experiments; error bars indicate standard deviations**

The cell viability of the cells grown for 96 h is shown in Fig. 2. Cell viability increased up to 12 h of fermentation, then decreased at 18 h, and remained at a similar level until the end of fermentation. This result is typical for yeast viability during fermentation, where cell viability usually increases during the first 6-12 h and then maintains a relatively steady level until it decreases at later time points, depending on the strain properties (Ishmayana *et al.*, 2015b).

The results of ethanol concentration analysis in basal fermentation of YEP medium are presented in Fig. 3. It revealed that the optimum fermentation time is 72 h, as the values for glucose content, ethanol content, OD, and cell viability showed no significant differences from 72 to 96 h. At 72 h, the ethanol content reached its maximum value, with an average of 37.12 mg $mL^{-1}$. According to LSD Fisher's post-hoc test, there was no significant difference in ethanol yield between 72, 84, and 96 h, indicating that 72 h is the optimum fermentation time. This suggests that yeast cell growth has entered the stationary phase.

***Fermentation yield***

To evaluate the accuracy of the predictive model, Table 2 presents a comparison between the experimental and predicted ethanol yields. The theoretical maximum ethanol yield from glucose in the study was approximately 0.511 g ethanol per g glucose (Pei *et al.*, 2024). In addition, the statistical significance of the model parameters is summarized in Table 3, which shows the ANOVA results from the RSM-CCD analysis and the mathematical equation obtained is shown in equation (1). The p-values for all individual metal ion concentrations were higher than 0.05, indicating that none of the individual metal ions significantly affected ethanol yield. However, the interaction between $Ca^{2+}$ and $Mg^{2+}$ ($p = 0.019$) and $Mg^{2+}$ and $Zn^{2+}$ ($p = 0.046$) showed a significant effect on ethanol yield, while the interaction between $Ca^{2+}$ and $Zn^{2+}$ ($p = 0.464$) was insignificant. This suggests that, while the individual effects of metal ions are insignificant, their interactions contribute in improving ethanol yield.

$$\text{Ethanol yield (g g}^{-1}) = -0.007 + 0.00260\ Ca^{2+} - 0.00033\ Mg^{2+} + 0.0124\ Zn^{2+} + 0.000087\ Ca^{2+}*Ca^{2+} - 0.000002\ Mg^{2+}*Mg^{2+} - 0.000224\ Zn^{2+}*Zn^{2+} - 0.000039\ Ca^{2+}*Mg^{2+} - 0.000106\ Ca^{2+}*Zn^{2+} + 0.000064\ Mg^{2+}*Zn^{2+} \quad \ldots\ldots (1)$$

To better understand the individual contributions of each metal ion to ethanol yield, the main effects of calcium, magnesium, and zinc concentrations were analysed. Fig. 4 presents the main effect plot of calcium, magnesium, and zinc ion concentrations on ethanol yield. For calcium ions, ethanol yield

**Table 2: Results of RSM-CCD runs with experimental and predicted fermentation efficiencies as responses**

| Run order | $Ca^{2+}$ conc. (mg $L^{-1}$) | $Mg^{2+}$ conc. (mg $L^{-1}$) | $Zn^{2+}$ conc. (mg $L^{-1}$) | Experimental ethanol yield (g $g^{-1}$) | Predicted ethanol yield (g $g^{-1}$) |
|---|---|---|---|---|---|
| 1 | 60.0 | 200.0 | 50.0 | 0.137 | 0.227 |
| 2 | 60.0 | 200.0 | 50.0 | 0.267 | 0.227 |
| 3 | 60.0 | 200.0 | 50.0 | 0.138 | 0.227 |
| 4 | 60.0 | 368.2 | 50.0 | 0.189 | 0.130 |
| 5 | 40.0 | 300.0 | 40.0 | 0.215 | 0.227 |
| 6 | 60.0 | 200.0 | 33.2 | 0.264 | 0.226 |
| 7 | 40.0 | 300.0 | 60.0 | 0.252 | 0.323 |
| 8 | 60.0 | 200.0 | 66.8 | 0.148 | 0.102 |
| 9 | 60.0 | 31.8 | 50.0 | 0.243 | 0.218 |
| 10 | 60.0 | 200.0 | 50.0 | 0.214 | 0.227 |
| 11 | 60.0 | 200.0 | 50.0 | 0.227 | 0.227 |
| 12 | 80.0 | 100.0 | 40.0 | 0.456 | 0.445 |
| 13 | 40.0 | 100.0 | 40.0 | 0.196 | 0.249 |
| 14 | 80.0 | 300.0 | 40.0 | 0.051 | 0.110 |
| 15 | 80.0 | 300.0 | 60.0 | 0.114 | 0.120 |
| 16 | 26.4 | 200.0 | 50.0 | 0.381 | 0.329 |
| 17 | 80.0 | 100.0 | 60.0 | 0.154 | 0.202 |
| 18 | 60.0 | 200.0 | 50.0 | 0.366 | 0.227 |
| 19 | 40.0 | 100.0 | 60.0 | 0.090 | 0.091 |
| 20 | 93.6 | 200.0 | 50.0 | 0.355 | 0.323 |

**Table 3: Analysis of variance (ANOVA) from the RSM-CCD design for the effects and interactions of calcium, magnesium, and zinc ions on ethanol yield**

| Source | DF | Adj SS | Adj MS | F value | p value |
|---|---|---|---|---|---|
| Model | 9 | 0.145772 | 0.016197 | 2.59 | 0.077 |
| Linear | 3 | 0.027786 | 0.009262 | 1.48 | 0.278 |
| $Ca^{2+}$ conc. | 1 | 0.000035 | 0.000035 | 0.01 | 0.942 |
| $Mg^{2+}$ conc. | 1 | 0.009218 | 0.009218 | 1.48 | 0.252 |
| $Zn^{2+}$ conc. | 1 | 0.018533 | 0.018533 | 2.97 | 0.116 |
| Square | 3 | 0.033131 | 0.011044 | 1.77 | 0.216 |
| $Ca^{2+}$ conc.*$Ca^{2+}$ conc. | 1 | 0.017513 | 0.017513 | 2.81 | 0.125 |
| $Mg^{2+}$ conc.*$Mg^{2+}$ conc. | 1 | 0.005137 | 0.005137 | 0.82 | 0.386 |
| $Zn^{2+}$ conc.*$Zn^{2+}$ conc. | 1 | 0.007241 | 0.007241 | 1.16 | 0.307 |
| 2-Way Interaction | 3 | 0.084855 | 0.028285 | 4.53 | 0.030 |
| $Ca^{2+}$ conc.*$Mg^{2+}$ conc. | 1 | 0.048984 | 0.048984 | 7.85 | 0.019 |
| $Ca^{2+}$ conc.*$Zn^{2+}$ conc. | 1 | 0.003612 | 0.003612 | 0.58 | 0.464 |
| $Mg^{2+}$ conc.*$Zn^{2+}$ conc. | 1 | 0.032258 | 0.032258 | 5.17 | 0.046 |
| Error | 10 | 0.062419 | 0.006242 | | |
| Lack-of-Fit | 5 | 0.025336 | 0.005067 | 0.68 | 0.657 |
| Pure Error | 5 | 0.037083 | 0.007417 | | |
| Total | 19 | 0.208191 | | | |

decreased up to approximately 60 mg $L^{-1}$ supplementation but increased again above that level. In contrast, magnesium and zinc ion concentrations showed a similar pattern, where ethanol yield increases up to a certain point (around 180 mg $L^{-1}$ for magnesium and 45 mg $L^{-1}$ for zinc) and then decreases thereafter. This indicates that to achieve high ethanol yield, the calcium ion concentration can be either below or above 60 mg $L^{-1}$, while magnesium and zinc ion concentrations should be maintained at approximately 180 mg $L^{-1}$ and 45 mg $L^{-1}$, respectively. However, when these ions are combined, the optimal concentrations may vary slightly due to their interaction effects, as previously described. Therefore, further analysis using a contour plot is required to determine the exact optimum conditions.

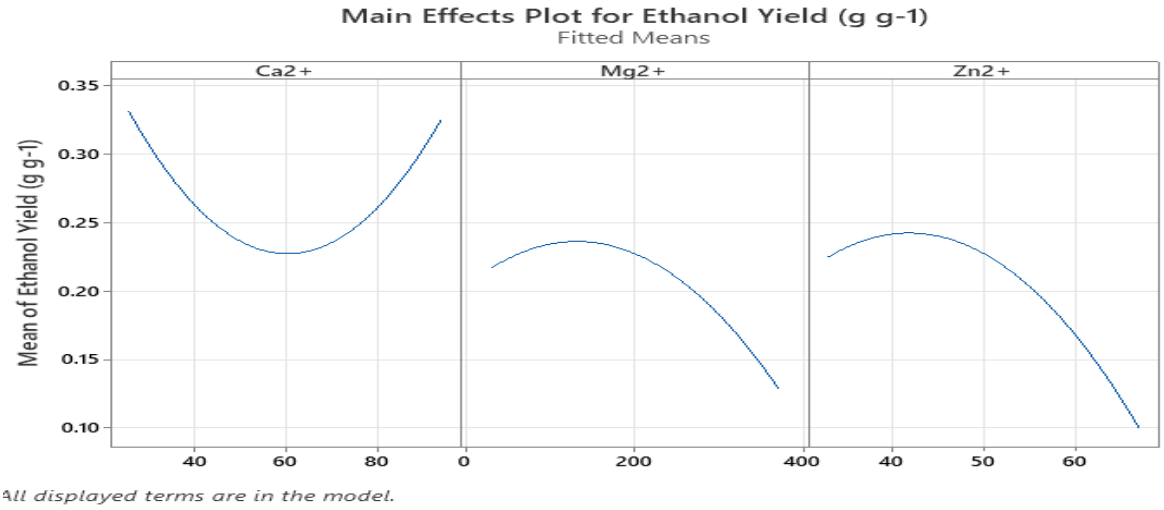


**Fig. 4: Main effect plot of calcium, magnesium, and zinc ions concentrations on ethanol yield**

***Contour plot of interaction between variables on ethanol yield***

Fig. 5 represents the contour plots of interaction illustrating how different combinations of calcium, magnesium, and zinc ions affected the ethanol yield. The plots display a saddle pattern, indicating that the optimum condition for achieving the highest ethanol yield is not explicitly visible. However, the highest ethanol yield (~0.5 g $g^{-1}$) can be achieved either at low calcium and high magnesium concentrations or at high calcium and low magnesium concentrations, represented by the dark green regions in the top-left and bottom-right corners of the plot (Fig. 5A). This suggests two possible optimum conditions.

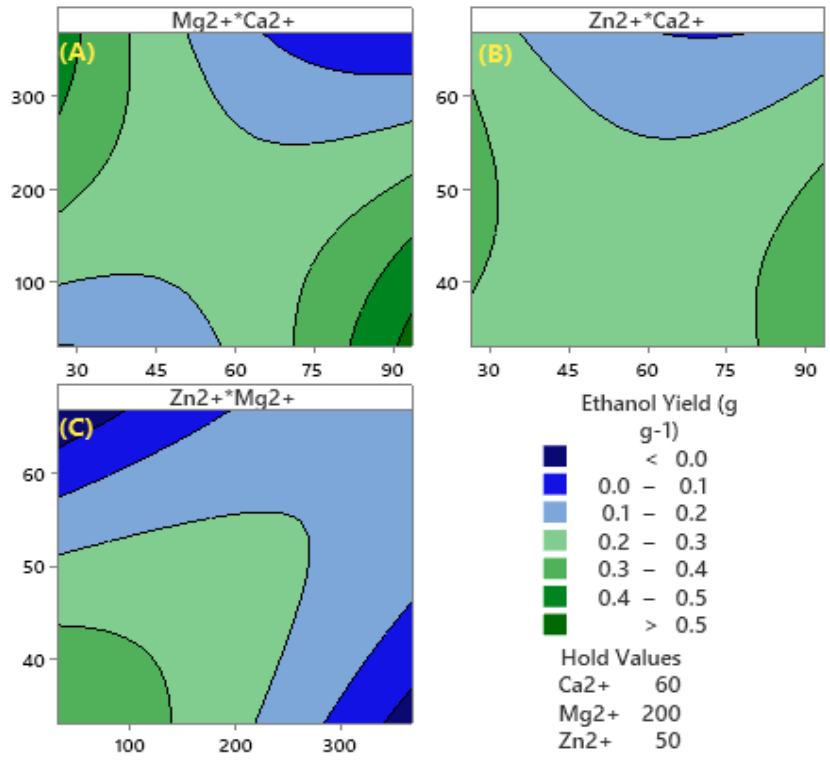


**Fig. 5: Contour plots showing the interaction effects of metal ions on ethanol yield (g $g^{-1}$): (A) calcium vs magnesium, (B) calcium vs zinc, and (C) magnesium vs zinc**

When the optimum condition was analyzed and five possible solutions were generated, the results were presented in Table 4. The optimum condition was selected based on the composite desirability value, where a value close to 1 indicated better optimization performance. Solutions 1 and 2 both had a composite desirability value of 1.0, indicating that they satisfy the optimization criteria. However, solution 1 predicted an ethanol yield (0.773 g $g^{-1}$) that significantly exceeded the

theoretical maximum value (0.511 g $g^{-1}$). Therefore, solution 2, which provided a more reasonable ethanol yield (0.567 g $g^{-1}$), was selected for validation.

**Table 4: Optimized solutions for calcium, magnesium, and zinc ion concentrations to maximize ethanol yield based on RSM-CCD results**

| Solutions | $Ca^{2+}$ conc. (mg $L^{-1}$) | $Mg^{2+}$ conc. (mg $L^{-1}$) | $Zn^{2+}$ conc. (mg $L^{-1}$) | Ethanol yield (g $g^{-1}$) | Composite desirability |
|---|---|---|---|---|---|
| 1 | 93.64 | 31.82 | 33.18 | 0.773 | 1.0000 |
| 2 | 26.36 | 368.18 | 66.82 | 0.567 | 1.0000 |
| 3 | 93.64 | 38.45 | 54.00 | 0.455 | 0.9972 |
| 4 | 26.36 | 330.12 | 51.94 | 0.449 | 0.9826 |
| 5 | 60.00 | 31.82 | 33.18 | 0.396 | 0.8511 |

Validation experiments were conducted to determine whether the suggested optimal solution aligns with the experimental results. The outcomes are presented in Table 5. The experimental ethanol yield (0.274 ± 0.018 g $g^{-1}$) was higher than the center point value (0.227 g $g^{-1}$) but significantly lower than the predicted value (0.567 g $g^{-1}$). This discrepancy may be attributed to several factors.

**Table 5: Validation of the optimal calcium, magnesium, and zinc ion concentrations based on RSM-CCD results**

| Conditions | $Ca^{2+}$ conc. (mg $L^{-1}$) | $Mg^{2+}$ conc. (mg $L^{-1}$) | $Zn^{2+}$conc (mg $L^{-1}$) | Ethanol yield (g $g^{-1}$) |
|---|---|---|---|---|
| Center point | 60.00 | 200.00 | 50.00 | 0.227 |
| Suggested optium condition | 26.36 | 368.18 | 66.82 | 0.567 |
| Validation Experiment 1 | | | | 0.283 |
| Validation Experiment 2 | 26.36 | 368.18 | 66.82 | 0.253 |
| Validation Experiment 3 | | | | 0.286 |
| | | | | 0.274 ± 0.018 |

Among the possible reasons for reduced ethanol yield during fermentation are the adverse effects of high initial glucose concentration and the evaporation of produced ethanol. Although the initial glucose concentration of 20% (w/v) is not classified as very high gravity fermentation, it is sufficient to increase the osmotic pressure of the medium, potentially affecting yeast performance and reducing ethanol production (Zaky *et al.*, 2020). Additionally, ethanol is volatile and readily evaporates due to its lower boiling point compared to water, which may lead to a reduction in the measurable ethanol yield at the end of fermentation (Agrawal, 2012). While the amount lost through evaporation is relatively small, it can still contribute to the overall yield discrepancy.

Furthermore, the RSM model is based on limited data points and assumes linear and quadratic relationships, which may not fully capture the complexity of biological systems (Taiwo and Musonge, 2023). Variability in the fermentation process such as fluctuations in pH, oxygen availability, or microbial activity can also influence yield and are difficult to control precisely (Lin *et al.*, 2012). In addition, experimental errors, including minor inconsistencies in inoculum size, ion concentrations, or analytical measurements, may also contribute (Akpoghelie *et al.*, 2024). Collectively, these factors could explain the observed gap between predicted and experimental ethanol yields.

In this study, ethanol yield was successfully enhanced by optimizing metal ion concentrations in YEP medium using RSM-CCD. The optimal concentrations of calcium, magnesium, and zinc ions were identified as 26.36, 368.18, and 66.82 mg $L^{-1}$, respectively. Under these conditions, ethanol yield reached 0.274 ± 0.018 g $g^{-1}$ i.e. 20.7% higher than the center point value, though still lower than the model's predicted yield. Compared to fermentation using YNB medium (Ishmayana *et al.*, 2025), the current results demonstrate that YEP medium supports a higher ethanol yield (0.274 vs. 0.197 g $g^{-1}$), suggesting that organic nitrogen sources more effectively enhance yeast fermentation performance. These findings underscore the importance of balancing metal ion interactions in the fermentation

medium to maximize ethanol production. The insights gained regarding optimal calcium, magnesium, and zinc ion concentrations may be valuable for improving ethanol yields in industrial fermentation systems, where medium formulation must balance nutrient supplementation with cost-effectiveness. Further studies are recommended to validate these results and refine medium composition to achieve even greater yields, ultimately contributing to more efficient bioethanol production processes.

Despite the discrepancy between experimental and predicted yields, the optimized metal ion concentrations identified in this study show promising potential for application in large-scale bioethanol production. In industrial settings, fine-tuning the balance of calcium, magnesium, and zinc ions could enhance fermentation efficiency and ethanol output, particularly under high-glucose conditions. However, practical implementation requires careful consideration of factors such as cost, the feasibility of ion supplementation at scale, and uniform nutrient distribution in large fermenters. Pilot-scale studies are recommended to evaluate the effectiveness of these optimized conditions in continuous or fed-batch systems commonly used in industry.

**Conclusion:** This study successfully identified the interaction effects of calcium, magnesium, and zinc ions on ethanol yield during high-glucose fermentation. The optimized concentrations, as determined through RSM, contributed to a notable improvement in ethanol production compared to the center point. However, a significant gap between predicted and experimental yields was observed, likely due to biological complexity, model limitations, and process-related factors such as ethanol evaporation and osmotic stress. These findings highlight the importance of refining process parameters and validating optimal ion concentrations under scaled-up conditions. Future research should focus on minimizing ethanol loss and improving model accuracy to enhance predictability and industrial applicability.

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**Authors contribution:** SI and MF designed the study, analysed the data, and revised the manuscript. DNU performed the experiments and prepared the initial draft of the manuscript. DTU analysed the data and contributed to drafting the manuscript. The manuscript was further refined with edits and suggestions from AS, FSN, RPF, and UMSS. All authors have read and approved the final version of the manuscript.

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