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ORIGINAL ARTICLE

Biorefinery potential of newly isolated yeast *Clavispora lusitaniae* for co-production of erythritol and ethanol

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Abstract

This study reports the biorefinery potential of a newly isolated yeast, *Clavispora lusitaniae* JARR-1, for production of erythritol as a sweet low-calorie food additive, along with co-production of ethanol as a biofuel candidate. The co-production process for synthesis of ethanol and erythritol was optimized using two-stage response surface methodology employing Placket-Burman (PB) and Central Composite Rotatable (CCR) designs. PB design indicated that the medium components, glucose, peptone, urea, and $MgSO_4$, significantly affected ($p < 0.05$) erythritol production. Furthermore, optimization of erythritol and ethanol co-production process by CCR design revealed the significant ($p < 0.05$) interactive effects of glucose and peptone on erythritol production and glucose, peptone, and $MgSO_4$ on ethanol production. Under optimized co-production process, maximum titers of erythritol and ethanol were 22.02 ± 0.04 g/L and 68.00 ± 0.02 g/L, respectively. The optimized process resulted in fivefold increase in erythritol production. Thus, *C. lusitaniae* JARR-1 has potential for sustainable co-production of erythritol and ethanol using biorefinery approach.

Highlights

- **Erythritol production by *Clavispora lusitaniae* JARR-1**
- **Erythritol and ethanol co-production optimization by RSM**
- **fivefold erythritol production (from 4.2 ± 0.19 to 22.02 ± 0.04 g/L) after optimization**
- **Glucose (20% wt.) & peptone (3% wt.) significantly affected erythritol production**
- **Maximum ethanol co-production of 68.00 ± 0.02 g/L**

Keywords Erythritol · *Clavispora lusitaniae* · Response surface methodology · Optimization · Ethanol · Biorefinery

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1 Introduction

Polyols (also known as a sugar alcohols, polyhydroxy alcohols, polyhydric alcohols, or polyalcohols) comprise a large group of low-digestible carbohydrates formed by the reduction of aldehyde or ketone groups [1]. These are predominantly valued for their role as sweeteners and bulking agents in food, particularly in bakery sector. Some examples of the polyols are lactitol, sorbitol, maltitol, mannitol, glycerol, xylitol, and erythritol. Erythritol, a C-4 sugar polyol, has received special attention in the food and pharmaceutical market due to its several advantages over sucrose, such as its noncaloric and noncariogenic sweetening property, low chemical reactivity, 20–40% less sweetness than sucrose, and safety for people with diabetes [2–4]. Moreover, the four-carbon backbone increases the prospects of erythritol

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as a platform chemical in biorefinery for the synthesis of many useful chemicals including 1,4-anhydroerythritol, 1,4-butanediol, butanetriol, butanol, 2,5-dihydrofuran, and tetrahydrofuran [2].

Microbial production of erythritol involves a wide variety of osmophilic yeasts belonging to *Trichosporonoides* sp., *Yarrowia* sp., *Candida* sp., *Moniliella* sp., etc. [5–7], bacteria such as *Leuconostoc oenos* [8], and some fungi, including *Pseudozyma tsukubaensis* [9], *Torula* sp. [10], and *Aureobasidium* sp. [11]. The biochemical route during microbial erythritol production involves pentose phosphate pathway (PPP) of carbon metabolism. During PPP, erythrose-4-phosphate is generated, which acts as the main precursor of erythritol. Dephosphorylation of erythrose-4-phosphate in subsequent steps leads to synthesis of erythrose. Erythrose is finally reduced to erythritol by the action of the enzyme erythrose reductase [12, 13]. PPP also plays an important role in cellulosic bioethanol production using glucose and/or xylose [14], since intermediates, such as glyceraldehyde-3-phosphate and fructose-6-phosphate, act as connecting link between glycolytic pathway for ethanol production and PPP.

At present, some industries such as Zevic, Urban Platter, and Herboveda India (https://zevic.in/; https://urbanplatter.in/product/urban-platter-pure-erythritol-powder-450g-all-natural-zero-gi-zero-calorie-sweetener/; https://www.herbovedaindia.com/Products/Erythritol/Index.shtml; last assessed in Oct 2021) are already producing and/or marketing bulk quantities of plant-based erythritol in India. Therefore, significant advancements in the microbial technology for erythritol production are required to improve process economics and provide competitive advantage over plant-based erythritol production. Large-scale and economic microbial production of erythritol is expected to avoid competition with plant-based food sources, making erythritol more acceptable as a biorefinery-based platform chemical, in addition to its current industrial applications. Technological advancements in erythritol production to lower the production costs can be potentially achieved by using following strategies, singly or in combination: (1) searching for more robust and high-yielding microorganisms, (2) improvement of existing erythritol producing microorganisms by random (conventional) mutagenesis, (3) developing tailor-made microorganisms with improved or new capabilities through recombinant DNA technology, (4) optimization of culture conditions and medium components by using statistical approaches, and (5) use of cheaper carbon feedstocks like waste glycerol and lignocellulosic biomass.

Earlier, the conventional mutagenesis with UV radiations and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was preferred for improving the erythritol production capabilities of *Candida magnoliae* [3] and *Moniliella* sp. 440 [15]. Genetic engineering-based methods are now increasingly being applied for improving erythritol fermentation capacities of yeasts [3, 15]. Examples of genetic engineering-based strategies include cloning and heterologous expression of the genes encoding three isozymes of erythrose reductases from *Trichosporonoides oedocephalis* [16], deletion of erythrulose kinase synthesis gene (EYK1) of *Y. lipolytica* to limit the metabolic uptake and subsequent utilization of secreted erythritol [17], and overexpression of transketolase gene in *Y. lipolytica* [18].

Statistical optimization of culture conditions and medium components is another useful strategy for enhancing erythritol production by wild-type or improved microbial strains [3, 19, 20]. It is carried out by using the response surface methodology (RSM), such as Placket-Burman (PB) design, Central Composite Rotatable (CCR) design, Box-Behnken (BB) design, etc. or the Taguchi design [20]. Statistical approach not only helps in enhancing the erythritol production with optimal use of resources (nutrients, medium components, environmental factors, etc.), but also provides information about significant parameters and their interactive effects on erythritol production. RSM based statistical optimization has been used for identifying and fine-adjustment the critical medium components for improved erythritol production by mutants of *C. magnoliae* NCIM 3470 [3]. Furthermore, supplementation of mineral ions (Cu^{2+}, Zn^{2+}, and Mn^{2+}) through RSM-based optimization has also been achieved to significantly enhance erythritol yield and productivity of *Y. lipolytica* [19].

Yet another useful but less commonly employed strategy for improving overall process economics is to co-produce another useful value-added product or chemical along with erythritol, such as bioethanol, enzyme, and organic acid. Presently, sustainable microbial biorefinery-based processes for multiple biochemical/bioproduct synthesis and potential to avoid the food-energy nexus are the subjects of global interests for research and development. Studies on erythritol co-production are very rare, and co-production of erythritol with lipase enzyme [5] or with citric acid [21, 22], using yeast *Y. lipolytica*, is the only available report so far. Erythritol and ethanol co-production has not yet received attention of researchers in the past, as there is lack of availability of such strains. Ethanol, produced by fermentation of sugars derived from hydrolysis of starch and lignocellulose, finds many industrial applications as a biofuel, solvent, and platform chemical. Therefore, industrial applications of the microorganisms co-producing erythritol and ethanol need to be explored. This co-production could contribute to significant reduction in the overall cost of production of these dual products in comparison to their individual production.

We had recently isolated an erythritol producing yeast *Clavispora lusitaniae* JARR-1, which also produced significant amounts of ethanol. The main objective of this study

was, therefore, to employ this yeast for co-production of erythritol and ethanol, and to optimize the co-production process using response surface methodology-based statistical optimization. The biorefinery potential of yeast *Clavispora lusitaniae* reported here for co-production of erythritol and ethanol can be beneficial in mitigating global challenge of food-energy nexus.

2 Materials and methods

2.1 Isolation and screening of erythritol producing microorganism

Various fruit, vegetable (apple pomace, citrus juice, sugarcane juice, cucumber, kinnow), and a few agro-industrial waste samples (whey, bagasse, and molasses) were collected from the local market of Mahendergarh, Haryana (28.2734° N, 76.1401° E), India. Ten grams of each sample was transferred separately in 90 mL of 0.85% (w/v) saline supplemented with kanamycin (30 µg/mL) for inhibiting bacterial growth, followed by incubation at 30 °C. After 5 days, aliquot from each sample was serially diluted and 100 µL of diluted sample was spread plated on yeast peptone dextrose agar (YPD) medium containing (%, w/v) glucose, 3; yeast extract, 1; peptone, 2; agar, 2; and kanamycin (30 µg/mL). After inoculation, the plates were incubated at 30 °C for 2 days. The morphologically distinct colonies appearing on the plates were purified and maintained on YPD plates having 20 g/L glucose at 4 °C. For primary screening, isolated yeast colonies were individually inoculated in 50 mL YPD broth, and the cultures were incubated at 30 °C and 200 rpm for erythritol production. After 4 days incubation, the samples were harvested, centrifuged at 10,000 rpm, and 4 °C for 10 min, and the supernatant was analyzed qualitatively for erythritol production using thin-layer chromatography (TLC). Quantitative estimation of erythritol produced by the yeasts was carried out by high-performance liquid chromatography (HPLC) analysis, and the best erythritol producing strain was selected for further exploration.

2.2 Phenotypic and phylogenetic characterization

The yeast cells were negatively stained using nigrosine black dye and observed under a light microscope at 100× magnification. Biochemical characterization involved HiCandida yeast identification kit (HiMedia Pvt. Ltd., India), following manufacturer's protocol. For molecular identification, genomic DNA was extracted using a previously described method with slight variations. Yeast cells from a day-old broth culture were harvested by centrifugation at 8,000 rpm for 10 min and resuspended in 200 µL lysis buffer composed (w/v) of 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris–HCl (pH 8.0), and 1 mM EDTA (pH 8.0). The suspension was then frozen in a dry ice-ethanol bath for 2 min, followed by thawed by immersing in a hot (95 °C) water bath for 1 min. The whole process of freezing and thawing was repeated twice, before vigorously vortexing for 30 s. This was followed by the addition of 200 µL chloroform, vortexing for 2 min and centrifugation at 12,000 rpm for 3 min. The aqueous layer was then transferred to a fresh microcentrifuge tube containing 400 µL ice-cold ethanol and allowed to precipitate for 5 min at room temperature, before another round of centrifugation at 12,000 rpm for 5 min. The obtained pellet containing the genomic DNA was air-dried, washed with 0.5 mL of 70% (v/v) ethanol, and then vacuum dried for 5 min at 60 °C. After drying, DNA was resuspended in 20 µL of Tris–EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and used as a template for polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS)-5.8S rDNA region with primer set: ITS1 (5′-TCCGTAGGT GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′). The PCR amplicon was sequenced on ABI 310 Genetic Analyzer (PE Applied Biosystems), commercially. For phylogenetic analysis, the obtained ITS-5.8S rDNA sequence was aligned with the similar sequences available in NCBI GenBank using the basic local alignment search tool (BLASTn) and ClustalW2 program. The phylogenetic tree was constructed using the MEGA software [23] employing the neighbor-joining method. The obtained ITS-5.8S rDNA sequence was submitted to GenBank.

2.3 Optimization of medium components for enhanced erythritol production

The effect of different glucose concentrations (100, 200, 300 g/L), various nitrogen sources (ammonium acetate, sodium nitrate, ammonium chloride, ammonium sulfate, urea, and peptone) at a fixed concentration of 1% (w/v), and two metal ions ($CaCl_2$ and $MgSO_4$) each at concentration of 20 mg/mL, on erythritol production by selected isolate, was studied initially by employing one factor at a time (OFAT) approach.

The statistical optimization of various parameters for enhanced erythritol production by the selected yeast was performed by two-tier RSM approach using Design-Expert software version 11 (Stat-Ease Inc., USA). In the first stage of RSM, screening of the most significant factors affecting erythritol production was carried out using PB design with 8 experimental runs. The variables selected in the PB design were concentrations of glucose, yeast extract, peptone, $CaCl_2$, $MgSO_4$, $NaNO_3$, and urea, along with three dummy variables. The most influential factors identified by PB design were considered at five levels each $(-\alpha, -1, 0, +1, +\alpha)$, during the second stage of RSM employing CCR design, and their effect on two responses, erythritol and ethanol concentrations (g/L),

Table 1 Central composite rotatable design matrix used for the optimization of erythritol production by *Clavispora lusitaniae* JARR-1, showing the coded (and actual) values of the dependent variables and the corresponding experimental responses, erythritol and ethanol

Standard order	Glucose (%, w/v)	Peptone (%, w/v)	Urea (%, w/v)	$MgSO_4$ (mg/L)	Erythritol (g/L)		Ethanol (g/L)	
					Actual value	Predicted value	Actual value	Predicted value
1	− 1 (20)	− 1 (2)	− 1 (2)	− 1 (20)	4.13 ± 0.08	4.27	35.0 ± 0.02	3.60
2	1 (40)	− 1 (2)	− 1 (2)	− 1 (20)	1.05 ± 0.04	2.11	82.4 ± 0.07	8.13
3	− 1 (20)	1 (3)	− 1 (2)	− 1 (20)	16.07 ± 0.02	17.01	69.5 ± 0.06	6.88
4	1 (40)	1 (3)	− 1 (2)	− 1 (20)	3.77 ± 0.09	5.27	84.0 ± 0.08	8.35
5	− 1 (20)	− 1 (2)	1 (3)	− 1 (20)	4.08 ± 0.07	4.70	59.5 ± 0.05	6.18
6	1 (40)	− 1 (2)	1 (3)	− 1 (20)	1.24 ± 0.05	2.34	99.3 ± 0.01	10.18
7	− 1 (20)	1 (3)	1 (3)	− 1 (20)	22.02 ± 0.04	20.00	68.0 ± 0.02	6.88
8	1 (40)	1 (3)	1 (3)	− 1 (20)	6.17 ± 0.06	8.08	79.1 ± 0.02	7.83
9	− 1 (20)	2 (− 1)	− 1 (2)	1 (40)	11.62 ± 0.03	10.98	61.3 ± 0.06	6.32
10	1 (40)	2 (− 1)	− 1 (2)	1 (40)	6.26 ± 0.01	8.64	87.5 ± 0.08	8.80
11	− 1 (20)	1 (3)	− 1 (2)	1 (40)	13.49 ± 0.03	12.75	76.8 ± 0.04	7.57
12	1 (40)	1 (3)	− 1 (2)	1 (40)	0.18 ± 0.08	0.83	71.2 ± 0.14	6.99
13	− 1 (20)	− 1 (2)	1 (3)	1 (40)	9.72 ± 0.01	8.58	68.2 ± 0.05	7.00
14	1 (40)	− 1 (2)	1 (3)	1 (40)	5.71 ± 0.08	6.05	87.9 ± 0.01	8.96
15	− 1 (20)	1 (3)	1 (3)	1 (40)	12.70 ± 0.06	12.92	54.6 ± 0.03	5.68
16	1 (40)	1 (3)	1 (3)	1 (40)	0.58 ± 0.06	0.80	45.6 ± 0.03	4.58
17	− 2 (10)	0 (2.5)	0 (2.5)	0 (30)	11.63 ± 0.08	13.75	44.7 ± 0.07	4.18
18	2 (50)	0 (2.5)	0 (2.5)	0 (30)	3.23 ± 0.09	-0.53	75.5 ± 0.08	7.61
19	0 (30)	− 2 (1.5)	0 (2.5)	0 (30)	2.03 ± 0.08	0.92	87.8 ± 0.06	8.37
20	0 (30)	2 (3.5)	0 (2.5)	0 (30)	8.95 ± 0.07	8.42	70.8 ± 0.05	7.26
21	0 (30)	0 (2.5)	− 2 (1.5)	0 (30)	8.88 ± 0.06	7.05	70.5 ± 0.03	7.23
22	0 (30)	0 (2.5)	2 (3.5)	0 (30)	7.25 ± 0.06	7.45	78.1 ± 0.04	7.39
23	0 (30)	0 (2.5)	0 (2.5)	− 2 (10)	14.89 ± 0.05	13.09	77.9 ± 0.05	7.73
24	0 (30)	0 (2.5)	0 (2.5)	2 (50)	12.36 ± 0.07	12.53	73.8 ± 0.08	7.20
25	0 (30)	0 (2.5)	0 (2.5)	0 (30)	15.31 ± 0.08	14.32	91.9 ± 0.05	9.29
26	0 (30)	0 (2.5)	0 (2.5)	0 (30)	13.18 ± 0.08	14.32	100.0 ± 0.06	9.29
27	0 (30)	0 (2.5)	0 (2.5)	0 (30)	15.51 ± 0.07	14.32	90.0 ± 0.04	9.29
28	0 (30)	0 (2.5)	0 (2.5)	0 (30)	15.78 ± 0.09	14.32	90.6 ± 0.02	9.29
29	0 (30)	0 (2.5)	0 (2.5)	0 (30)	13.18 ± 0.06	14.32	91.2 ± 0.02	9.29
30	0 (30)	0 (2.5)	0 (2.5)	0 (30)	14.07 ± 0.07	14.32	91.9 ± 0.05	9.29
31	0 (30)	0 (2.5)	0 (2.5)	0 (30)	13.18 ± 0.08	14.32	94.6 ± 0.01	9.29

was studied. The CCR design of experiments has been shown in Table 1.

The optimum values of selected variables were calculated from the regression equation (Eq. 1) and also derived from the analysis of the three-dimensional response surface contour plots.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j \quad (1)$$

where y is the response variable; x is the independent variable; β_0 is the constant term; β_i, β_{ii}, and β_{ij} are the regression coefficients of the linear, interaction, and quadratic parameters, respectively; and k is the number of variables studied.

All the optimization experiments were conducted in the YPD broth medium, having the composition as per the design of experiments by inoculating with the yeast culture and incubating at 30 °C and 200 rpm for 5 days. The harvested samples (5 mL) were centrifuged at 10,000 rpm and 4 °C for 10 min and the supernatant was quantitatively analyzed for erythritol, glucose, and ethanol by HPLC analysis.

2.4 Analytical methods

The qualitative detection of erythritol was carried out by TLC using a solvent system composed of ethyl acetate, acetone, chloroform, and ethanol in the ratio of 1:1:1:1 by volume. Polyols were stained and detected by spraying Molisch reagent

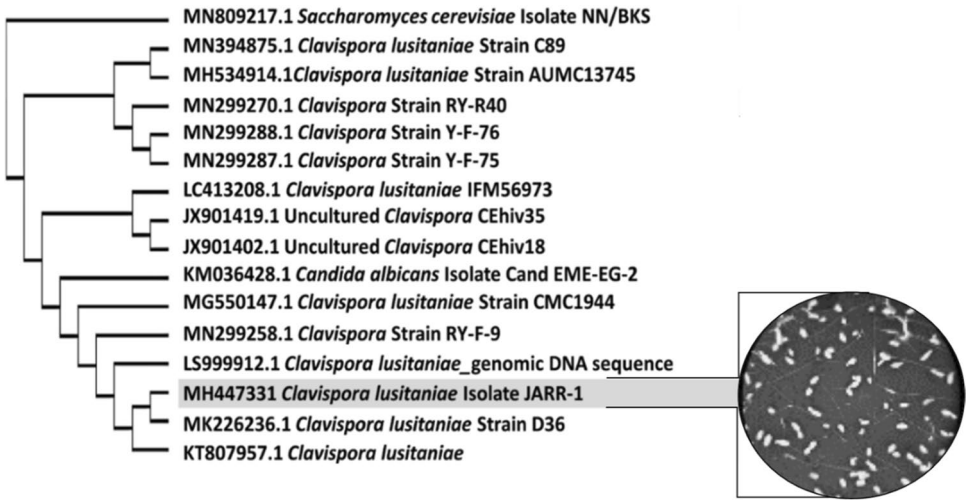

Fig. 1 Phylogenetic relationship of *Clavispora lusitaniae* JARR-1 with other related yeasts. The phylogenetic tree was made using MEGA software with the neighbor-joining method. The number of bootstrap replicates considered was 1000. The value is the proportion of replicate phylogenies that recovered a particular clade from the original phylogeny that was built using the original alignment. *Saccharomyces cerevisiae* NN/BKS was used as the outgroup. Inset shows a light photomicrograph of negatively stained budding cells (100 × magnification) of *C. lusitaniae* JARR-1

(10% α-naphthol and 5%, v/v H_2SO_4 in absolute ethanol) and heating at 120 °C for 10 min. The concentrations of erythritol and ethanol were determined by Adept CE 4800 series (Cecil, UK) HPLC system fitted with refractive index detector and 300 × 7.8 mm Aminex HPX-87H column (Bio-Rad, Hercules CA, USA) using 0.02 M H_2SO_4 prepared in Milli-Q water as an eluent (flow rate 0.6 mL/min) at room temperature. The reducing sugars were analyzed by the 3,5-dinitrosalicylic acid (DNS) method [24], using anhydrous glucose as standard. The cell density was determined by analyzing absorbance at 600 nm. All the experiments were conducted in triplicates, and the average values were reported.

3 Results and discussion

3.1 Isolation, screening, and characterization of new yeast strain

Environmental samples that are rich in nutrients (especially with high carbohydrate content) and have a pH in acidic range are ideal starting material for isolation of erythritol producing osmophilic yeasts [5, 6, 9]. In this study, various fruit, vegetable, and agro-industrial waste samples were utilized for the isolation of a total of 79 yeasts, out of which 25 distinct yeasts were producing erythritol as determined by TLC analysis. Secondary screening of these cultures for erythritol production, as estimated by HPLC analysis, revealed that the yeast named JARR-1 isolated from apple pomace displayed significantly higher ($p < 0.05$) erythritol production (4.01 g/L ± 0.18) than the rest of the isolates. Previous studies have also reported the isolation of the osmophilic erythritol producing yeasts from different agro-food wastes [25–27].

Molecular characterization of the yeast JARR-1 through PCR amplification and sequencing of 304 bp long ITS-5.8S rDNA sequence [28] revealed maximum similarity with ITS region of *Clavispora lusitaniae* (Fig. 1). The obtained sequence was submitted to GenBank under accession number MH447331 and the isolate was named as *Clavispora lusitaniae* JARR-1. It had typical yeast morphology with globose to spheroidal cells showing multilateral budding under the light microscope, as reported earlier for *Clavispora* sp. [28]. Multilateral budding was also reported previously in erythritol producing yeast *Pseudozyma tsukubaensis* isolated from sludge [11]. *C. lusitaniae* JARR-1 fermented glucose and utilized the sugars, maltose, rhamnose, sucrose, and xylose as well as glycerol and ethanol. The yeast neither produced urease enzyme nor utilized the sugars melibiose, lactose, galactose, cellobiose, inositol, dulcitol, raffinose, and trehalose, for its growth. The carbohydrate fermentation and growth pattern revealed typical biochemical characteristics of *Clavispora lusitaniae*, as described earlier [28].

3.2 Optimization of medium components for enhanced erythritol production

During preliminary OFAT optimization of erythritol production using JARR-1, maximum erythritol production (4.2 ± 0.19 g/L) was observed after 5 days incubation at 30 °C, in YPD medium having pH 5.0 to 7.5 and inoculum

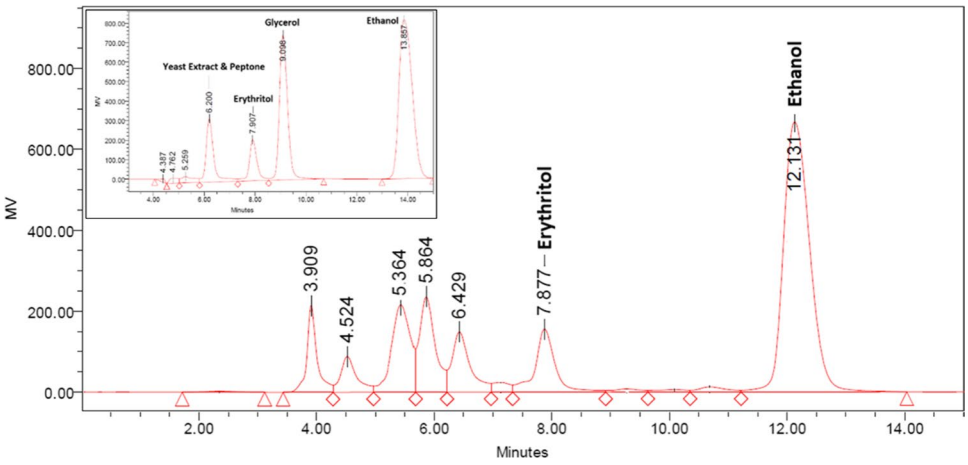

Fig. 2 HPLC chromatogram showing erythritol and ethanol production by *Clavispora lusitaniae* JARR-1. Inset shows the peaks of standards erythritol, ethanol, and glycerol

of 5%, v/v (from 18-h old culture). Therefore, these parameters were fixed for further experiments during the subsequent RSM-based optimization. PB design of experiments helped in determining the effect of individual parameters (various constituents of fermentation medium) on erythritol production and selecting the most significant factors for further optimization. The chromatogram in Fig. 2 depicts the production of erythritol and ethanol. The standardized effect of significant factors affecting erythritol production is shown in Fig. 3. The Pareto chart (Fig. 3a) and the normal plot (Fig. 3b) for PB design indicated that out of seven factors, four factors, namely concentrations of glucose, $MgSO_4$, urea, and peptone, had standardized effect values greater than the critical value 2.18 and hence significantly affected ($p < 0.05$) erythritol production. The significance of carbon and nitrogen sources and metal ion, in comparison to other medium components for erythritol production in this study, was in sync with previous reports [4], where urea [4], peptone [29], and metal ions [19] were reported to significantly elevate erythritol production by yeast *Y. lipolytica*. Glucose fermentation ability of *C. lusitaniae* is a well-known physiological and biochemical characteristic [28]. It has been reported that at high initial concentration of glucose, erythritol production by osmophilic yeasts is also higher [29, 30]. This might be due to the fact that enhancing the initial glucose concentration increases the erythritol production rate and yield in a batch process, if the microbes can tolerate a higher sugar concentration and a higher osmotic pressure. But, knowledge about the molecular mechanisms underlying the induction of such pathways [30]. A mutant of *Aureobasidium* was used to produce erythritol at 1.8 g/L/h with a 44% yield in a medium containing 400 g/L glucose [31]. The nitrogenous compound urea, besides maintaining the growth, also helps osmophilic yeasts resist osmotic stress during erythritol formation. The peptone has been reported as a potent nitrogen source for erythritol biosynthesis as its low concentration enhances the erythritol production [31]. Previous reports have also demonstrated the effect of metal ions on erythritol production during microbial fermentation, e.g., Mn^{2+} at 38 mg/mL enhances erythritol production due to increased cell permeability [10], while Ni^{2+} (> 20 mg/mL) inhibits its production [33]. Also, the addition of Cu^{2+} and Al^{3+} in culture medium increases erythritol biosynthesis [32].

Since, PB design could only help identify the critical medium components significantly influencing erythritol synthesis, without providing any information on the interactions among the selected critical parameters, it was therefore necessary to employ CCR design of RSM to reveal the interactive effects of critical parameters during erythritol production.

The employed CCR design was a four-factor, five-level design matrix having 31 experimental runs, resulting in wide variations in the values of the selected responses, i.e., concentrations of erythritol and ethanol (Table 1). Maximum erythritol was obtained in the run #07, where the optimized conditions resulted in erythritol and ethanol concentrations of 22.02 ± 0.04 and 68.0 ± 0.02 g/L, respectively. However, the maximum ethanol concentration of $100.00 + 0.06$ g/L was obtained in run #26. The observed values for erythritol and ethanol production were in agreement with the respective predicted values (Fig. 4 a and b). Erythritol production in this study was better than that shown by *Aureobasidium pullulans* mutant ER35 (17.28 g/L) [35], and was comparable to 23–24 g/L erythritol production by wild-type yeasts *Trigonopsis variabilis* KCCM 35,523 [35] and *Candida magnoliae* Mutant M2 [14]. However, erythritol production in this study was lower than the reported maximum production of 245, 166, and 31.75 g/L by the improved and high-yielding industrial yeast strains of *P. tsukubaensis* [11], *Torula* sp. [13], and *Aureobasidium* sp. [12], respectively. These high-yielding yeast strains have been developed after many years of efforts by improving their genetic capabilities, whereas the present study employed only the wild-type yeast

without any strain improvement. Nevertheless, the unavailability of previous reports on exploitation of *C. lusitaniae* for erythritol production, along with its ability to co-produce ethanol in good quantity (above the industrial benchmark of 40 g/L), indicates its dual advantage for biorefinery applications, thereby making the findings of this study interesting. Additionally, *Clavispora* sp. has been previously reported as a robust yeast having potential to grow with a high rate, and the ability to ferment sugar to ethanol while also producing a key cellulolytic enzyme, i.e. β-glucosidase [36]. This proves the potential candidature of this yeast in advanced biofuel production from lignocellulosic biomass. The indigenous production of β-glucosidase could be highly crucial for faster and cost-effective simultaneous saccharification and fermentation (SSF) of lignocellulosic biomass, as the plant biomass hydrolysis will only require cellulases supplementation without the need to supply external β-glucosidase. In the same study, it has also been reported to exhibit faster bioconversion rate of 0.088 g/L/h, for bioethanol production from plant biomass. Current study resulted in much higher production of erythritol by *Clavispora* sp. than earlier description of 10.9 g/L [36], whereas it was lower than 44.5 g/L reported previously [38].

3.3 The analysis of variance (ANOVA)

The interaction effects of medium components were estimated from the regression coefficients, *F*-values, and *p*-values of variables obtained during ANOVA for the models developed for erythritol and ethanol production by CCR design, as shown in Tables 2 and 3, respectively. Both the models were significant ($p < 0.01$), with model *F*-values of 18.29 and 55.45, respectively. There was only 0.1% chance that model *F*-values these large could occur due to noise. The lack of fit for both the developed models was non-significant, and the values of the coefficient of determinations ($R^2_{erythritol} = 0.94$ and $R^2_{ethanol} = 0.98$) were close to 1, indicating a good agreement between the experimental data and the model [38]. ANOVA indicated the fitness of both the models for prediction, as shown by the close match between the pre-determined $R^2_{ethanol} = 0.92$ and the adjusted $R^2_{ethanol} = 0.96$ and the reasonable agreement between the pre-determined $R^2_{erythritol} = 0.70$ and the adjusted $R^2_{erythritol} = 0.89$.

During regression analysis, the "prob > F" values of model terms less than 0.05 indicated their significant effect on the responses at 95% confidence level and the values greater than 0.1 pointed towards their non-significance. Based on "prob > F" values, the non-significant factors for erythritol production (C, D, D^2, AC, BC, CD, and AD) and for ethanol production (C and AC) were excluded from the respective models by data reduction. Thus, the following second-order quadratic equations for the responses erythritol (g/L) (Eq. 2) and ethanol concentrations (Eq. 3) were obtained after the regression analysis of the CCR design data:

$$\begin{aligned}\text{Erythritol (g/L)} = {} & 14.31 - 3.57A \\ & + 1.87B - 1.93A^2 - 2.41B^2 \\ & - 1.77C^2 - 0.38D^2 - 2.39AB + 0.64BC \\ & - 2.74BD - 0.74CD \end{aligned} \quad (2)$$

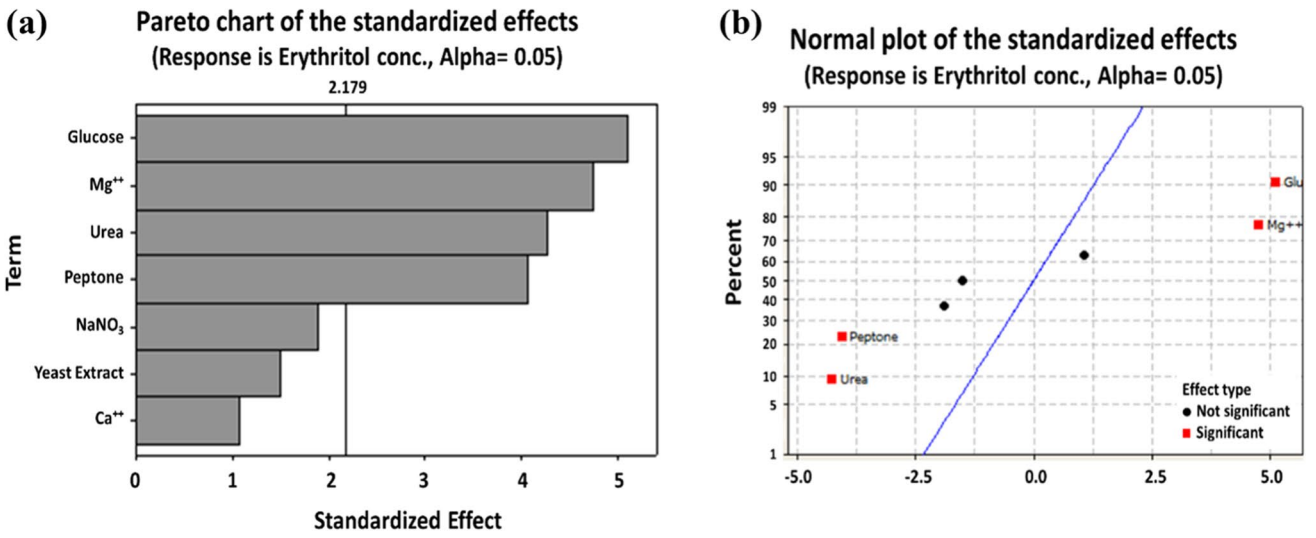

Fig. 3 Pareto chart (**a**) and normal plot (**b**) of the standardized effect showing significant factors for erythritol production. Mg^{2+} & Ca^{2+} denote $MgSO_4$ and $CaCl_2$, respectively

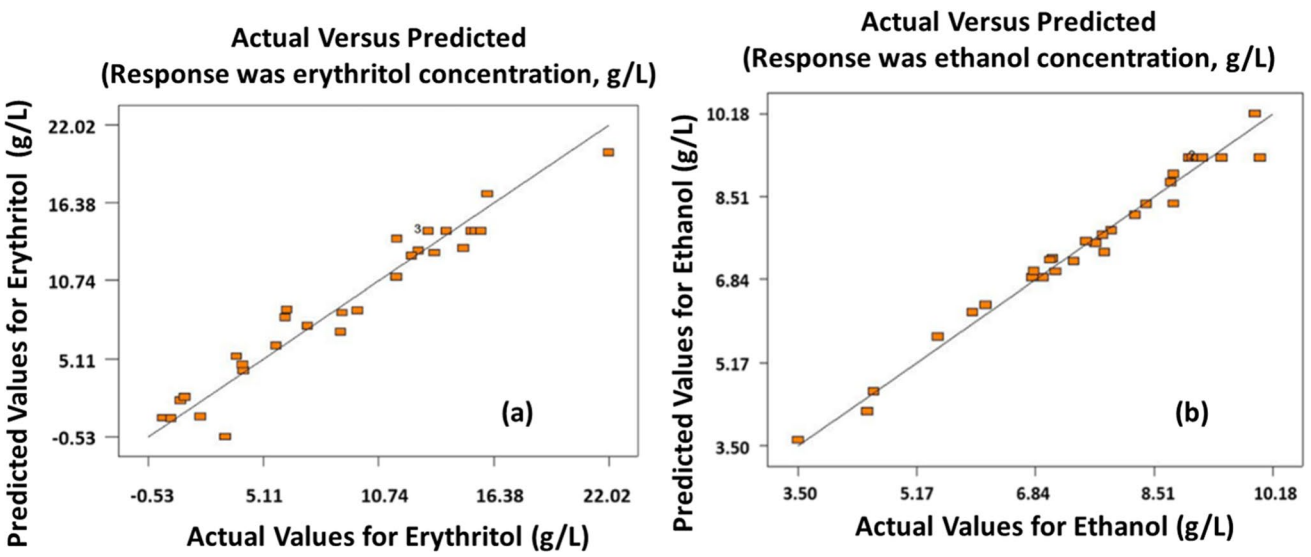

Fig. 4 The plot of residuals showing predicted vs. actual erythritol (**a**) and ethanol (**b**) production by *Clavispora lusitaniae* JARR-1

$$\text{Ethanol (g/L)} = 9.29 - 0.86A - 0.28B - 0.85A^2 - 0.37B^2 - 0.49C^2 - 0.46D^2 - 0.76AB - 0.51AD - 0.64BC - 0.50BD - 0.47CD \quad (3)$$

where A, B, C, and D were the independent variables, representing the concentrations of glucose (%, w/v), peptone (%, w/v), urea (%, w/v), and $MgSO_4$ (mg/L), respectively.

Table 2 Analysis of variance (ANOVA) of the quadratic model for erythritol production

Source	Sum of squares	DF	Mean square	*F*-value	Prob > *F*	
Model	918.47	14	65.61	18.29	< 0.0001	Significant
A	305.67	1	305.67	85.24	< 0.0001	
B	84.381	1	84.38	23.53	0.00	
C	0.2408	1	0.24	0.07	0.80	
D	0.4686	1	0.47	0.13	0.72	
A^2	106.16	1	106.16	29.60	< 0.0001	
B^2	166.29	1	166.29	46.37	< 0.0001	
C^2	89.345	1	89.34	24.91	0.00	
D^2	4.0591	1	4.06	1.13	0.30	
AB	91.65	1	91.65	25.56	0.00	
AC	0.0365	1	0.04	0.01	0.92	
AD	0.0337	1	0.03	0.01	0.92	
BC	6.5819	1	6.58	1.84	0.19	
BD	120.45	1	120.45	33.59	< 0.0001	
CD	8.0027	1	8.00	2.23	0.15	
Residual	57.378	16	3.59			
Lack of fit	48.868	10	4.89	3.45	0.07	Not significant
Pure error	8.5098	6	1.42			
Cor total	975.85	30				
Std. dev	1.89		$R^2_{erythritol}$		0.94	
Mean	9.30		Adj $R^2_{erythritol}$		0.89	
C.V	20.37		Pred $R^2_{erythritol}$		0.70	
PRESS	293.06		Adeq precision		15.58	

*Independent variables A, B, C, and D represent the concentrations of glucose (%, w/v), peptone (%, w/v), urea (%, w/v), and $MgSO_4$ (mg/L), respectively

3.4 Three-dimensional response surface analysis

Three-dimensional response surface curves for erythritol and ethanol production during CCR design are shown in Figs. 5 and 6, respectively, indicating the interaction of the two variables at a time while maintaining the remaining factors constant at their central values. The 3-D surface plots were used to determine the optimum level of each of the variable for maximum response. The coordinates of the central point within the highest contour levels in each of the figures corresponded to the optimum concentrations of the respective component, while the shapes of 3D plots indicated the nature and extent of the interactions.

The interactive effects of peptone and glucose, at the central value of urea and $MgSO_4$ (Fig. 5a) indicated that erythritol production was more when peptone and glucose were at higher and lower levels, respectively. A similar trend in dependence of erythritol concentration on higher urea and lower glucose levels was also observed (Fig. 5b); however, glucose at higher level had a profound negative impact on erythritol concentration. It was observed during the interaction of $MgSO_4$ and glucose (Fig. 5c) that the maximum erythritol production, i.e., 14.80 g/L, was obtained at lower level of $MgSO_4$, irrespective of the levels of the glucose. Glucose affected erythritol production negatively at either of the levels. In case of interaction of urea and peptone, maximum erythritol concentration was obtained near the intermediate levels of both the variables (Fig. 5d), whereas the higher as well as the lower extremes of levels of both the variables corresponded to a decline in erythritol concentration. Interaction of $MgSO_4$ with peptone or with urea had nearly similar effect on the response, resulting in higher erythritol concentrations either at lower level of $MgSO_4$ and higher level of peptone (Fig. 5e) or at lower level of $MgSO_4$ and urea (Fig. 5f). However, the comparative interactive effect of $MgSO_4$ and peptone on erythritol synthesis was highly positive, with a highest erythritol concentration of 20.73 g/L.

Figure 6a illustrates the calculated response surface from the interaction between peptone and glucose concentration. The interaction between peptone and glucose had a marginal positive effect on ethanol concentration, but increasing the peptone or glucose concentrations individually increased the ethanol concentration more. Highest ethanol concentration (90 g/L) was found at the lowest level of the peptone concentration, i.e., 2.25 g/L. Figure 6b shows the effect of interaction of urea and glucose on ethanol production. An increase in both glucose and urea up to the highest levels had a positive effect on ethanol concentration (10%, v/v). The effect of interaction between $MgSO_4$ and glucose on

Table 3 Analysis of variance (ANOVA) of the quadratic model for ethanol production

Source	Sum of squares	DF	Mean square	F value	Prob > F	
Model	77.75	14.00	5.55	55.45	< 0.0001	Significant
A	17.64	1.00	17.64	176.12	< 0.0001	
B	1.82	1.00	1.82	18.20	0.00	
C	0.04	1.00	0.04	0.39	0.54	
D	0.42	1.00	0.42	4.22	0.05	
A^2	20.63	1.00	20.63	205.96	< 0.0001	
B^2	3.89	1.00	3.89	38.82	< 0.0001	
C^2	6.98	1.00	6.98	69.74	< 0.0001	
D^2	5.95	1.00	5.95	59.37	< 0.0001	
AB	9.33	1.00	9.33	93.12	< 0.0001	
AC	0.27	1.00	0.27	2.74	0.12	
AD	4.19	1.00	4.19	41.79	< 0.0001	
BC	6.61	1.00	6.61	66.01	< 0.0001	
BD	4.10	1.00	4.10	40.91	< 0.0001	
CD	3.57	1.00	3.57	35.61	< 0.0001	
Residual	1.60	16.00	0.10			
Lack of fit	0.88	10.00	0.09	0.73	0.68	Not significant
Pure error	0.72	6.00	0.12			
Cor total	79.35	30.00				
Std. dev	0.32		$R^2_{ethanol}$		0.98	
Mean	7.61		Adj $R^2_{ethanol}$		0.96	
C.V	4.16		Pred $R^2_{ethanol}$		0.92	
PRESS	6.06		Adeq Precision		29.87	

*Independent variables A, B, C, and D represent the concentrations of glucose (%, w/v), peptone (%, w/v), urea (%, w/v), and $MgSO_4$ (mg/L), respectively

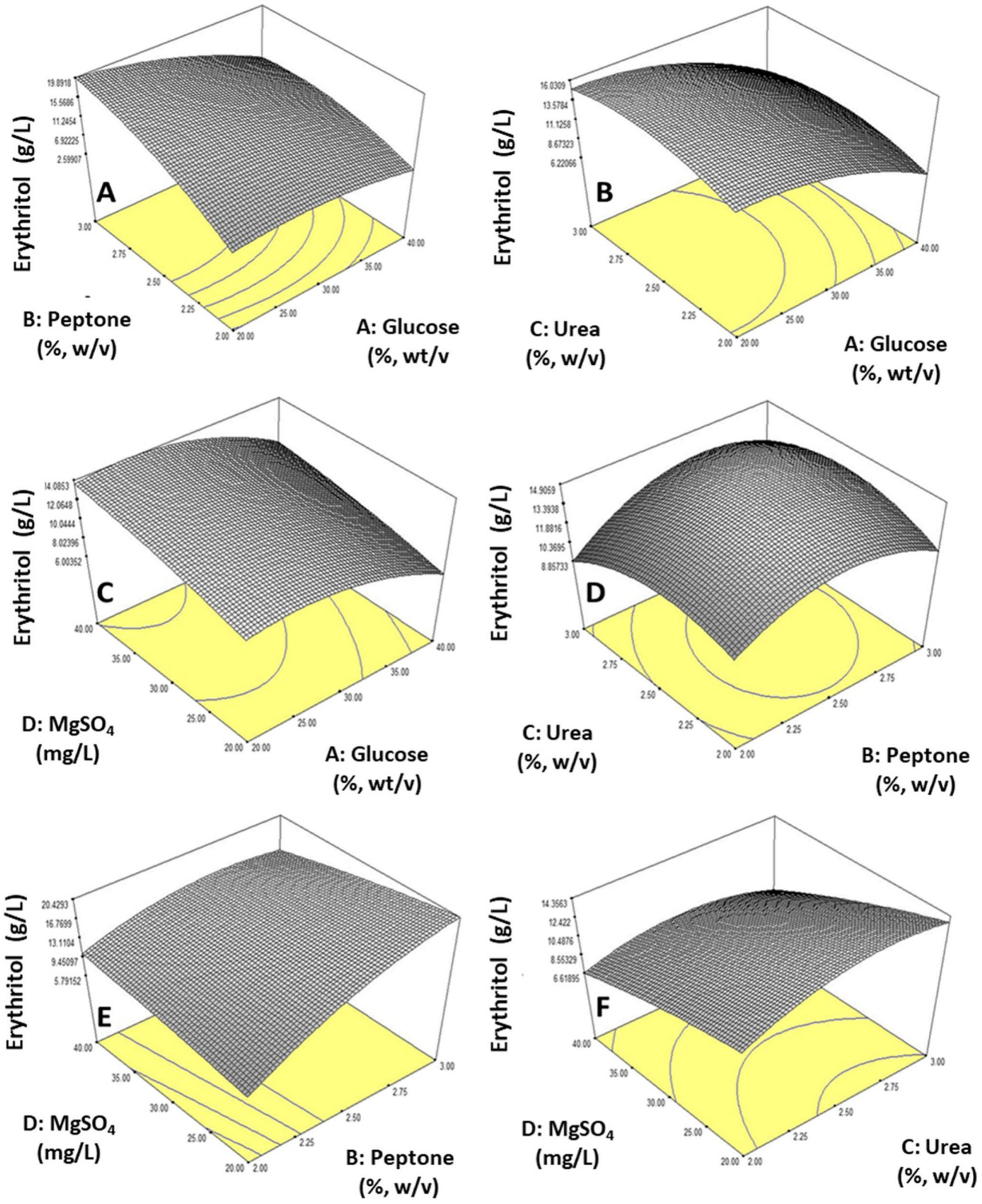

Fig. 5 Three-dimensional response surface plots showing the interaction of variables during erythritol production by *Clavispora lusitaniae* JARR-1

ethanol concentration is displayed in Fig. 6c. An increase in level of glucose and decrease of $MgSO_4$ concentration enhanced ethanol production. However, the effect of interaction between urea and peptone displayed that lower concentration of both resulted in higher ethanol concentration (Fig. 6d). Figure 6 e depicts the effect of interaction between $MgSO_4$ and peptone on ethanol concentration. The increase in concentration of both $MgSO_4$ and peptone did not yield much variations in ethanol concentration. The effect of interaction between $MgSO_4$ and urea was a positive increase in ethanol concentration at lower levels of $MgSO_4$ and higher levels of urea concentrations (Fig. 6f).

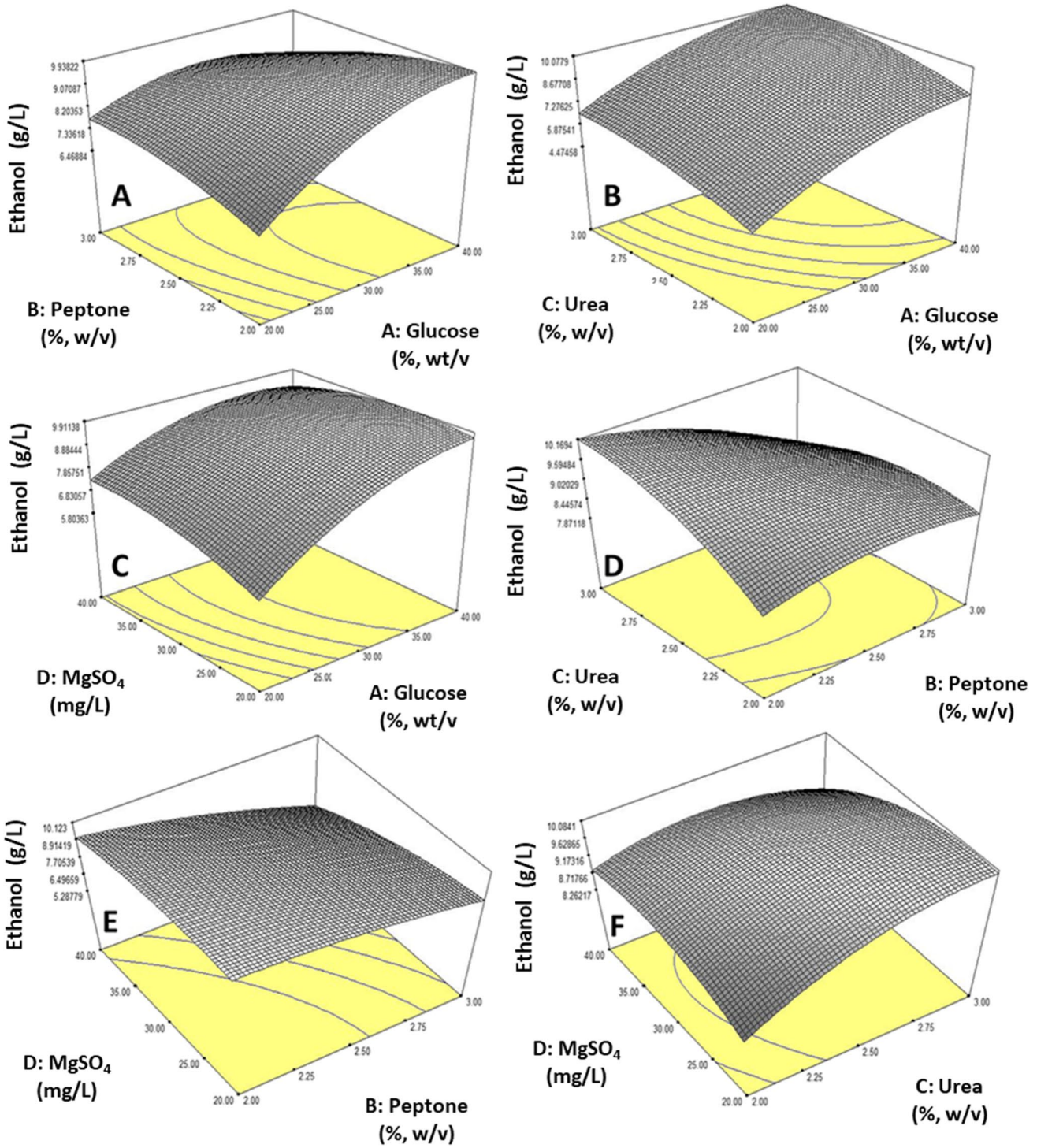

Fig. 6 Three-dimensional response surface plots showing the interaction of variables during ethanol production by *Clavispora lusitaniae* JARR-1

After point prediction, the optimized levels of parameters under consideration for erythritol production were determined as glucose, 20% (w/v); peptone, 3% (w/v); urea, 2.75% (w/v); and $MgSO_4$, 20 mg/L. Under optimized conditions, the erythritol concentration was 22.02 ± 0.04 g/L, whereas the concentration of ethanol under these conditions was 68.00 ± 0.02 g/L. The optimized medium was also used for erythritol production at 2 L shake flask level and similar titer of erythritol (21.8 ± 0.16 g/L) after 5 days was obtained. During erythritol production, *C. lusitaniae* JARR-1 utilized glucose almost completely and did not produce glycerol as determined by HPLC analysis. Previously, RSM-based media optimization had improved erythritol production by 1.54-fold (~ 32 g/L) in *A. pullulans* [4 and 1.25 to fivefold in *Candida magnolia* [3]. Conclusively, a total of fivefold improvement in erythritol production was attained in this study after the statistical optimization of the production medium.

Considering the increasing demand for biorefinery operations and their crucial roles in sustainable development, the results of the present study represent an important milestone in co-producing erythritol and ethanol as the biochemical products having food and fuel applications, respectively, which could be promising in avoiding the food-energy nexus to some extent. Literature survey indicates lack of previous reports on erythritol and ethanol co-production. In fact, erythritol co-production with other products has rarely been reported, and the only reported studies are of co-production of erythritol and lipase enzyme [5] and erythritol and citric acid [21], using yeast *Y. lipolytica*. Although, a few reports are available on ethanol production by *Clavispora lusitaniae* from glucose [33, 39], molasses, and sugars obtained by hydrolysis of the plant biomass such as wheat straw [40, 41], there is hardly any report so far on erythritol production by this yeast.

Due to higher demands of both erythritol and ethanol, co-production of these dual chemicals in future via fermentation of sugars derived from varied sources, such as starch or lignocellulose, would be a cost-effective strategy for reducing the overall cost of their individual production.

4 Conclusions

This study reported a new indigenously isolated erythritol producing yeast *Clavispora lusitaniae* JARR-1. This yeast produced maximum titers of erythritol 22.02 ± 0.04 g/L, along with the ethanol 68.00 ± 0.02 g/L, after statistical optimization of the co-production process. The optimized levels of medium components for erythritol production were glucose, 20% (w/v); peptone, 3% (w/v); urea, 2.75% (w/v); and $MgSO_4$, 20 mg/L, with a fermentation period of 5 days. Due to high biorefinery potential of *C. lusitaniae* JARR-1 for erythritol and ethanol co-production and considering limited reports on such co-production process, future studies on its strain improvement for developing robust cell-factories and further process scale-up will help in mitigating the food-energy nexus.

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Declarations

Conflict of interest The authors declare no competing interests.

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