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Application of Saccharomyces cerevisiae isolated from industrial effluent for zinc biosorption and zinc-enriched SCP production for human and animal

Saraei FOROUGH[1], Amini KUMARSS[2*], Haddadi AZAM[1], Larypoor MOHADDESEH[3]

Abstract

We aimed to optimize the culture condition for industrial effluent isolated *Saccharomyces cerevisiae* strains to reach the highest zinc biosorption, biomass, and protein production for human and animals. *S. cerevisiae* strains carrying ZRT and FET genes were isolated from effluent samples and identified using standard methods. Subsequently, the growth rate of yeasts in the presence of Zn2+, as well as the level of Zn2+ uptake by the yeast cells, were examined at 24-hour intervals. RT-PCR technique was applied to quantify the expression level of the target genes in yeast cells. The effect of the initial pH of culture medium was studied on the yeast growth rate, zinc absorption, and target genes expression. After setting the optimum pH, Kjeldahl method was applied for assessment of the total protein content of yeast cells. In the optimum conditions, *S. cerevisiae* showed the maximum growth rate, zinc uptake, and expression level of Zrt1 and Fet4. In addition, protein content of *S. cerevisiae* biomass in this optimum condition was above 50% (w/w). We demonstrated that *S. cerevisiae* species isolated from industrial effluents could be considered as highly promising candidates for producing Zn-enriched single cell protein. However, further research is believed to be required.

Keywords: *S. cerevisiae*; Zinc; biosorption; single cell protein.

Practical Application: The potential use of microorganisms for protein production.

1 Introduction

Numerous reports have indicated that air, soil, and water pollution with toxic and dangerous chemicals from rapidly developing industrial activities has posed high risks to living organisms, particularly human beings (Briffa et al., 2020). Various industries, such as chemical, pesticides, food, textile, and metallurgical industries, release large amounts of waste, containing metals, into the environment. Today, heavy metals are among the most important industrial pollutants in the environment, which could also affect human lives (Ukah et al., 2019). Among heavy metals, zinc, which is one of the basic elements in the structure of enzymes, is of great importance. This heavy toxic metal is often found along with iron and copper in effluents from various industrial units (Shifaw, 2018). On the other hand, zinc, as an essential element, has several important biological impacts, including being a major ion in the structure of motifs, acting as a catalytic factor of enzymes, having an essential role in the structure and function of nucleic acid and protein, and involving in gene expression and immune system development (Obasi & Akudinobi, 2020). Studies have shown that zinc deficiency results in numerous disorders, such as growth retardation and impaired immune function. In this background, biosorption strategies have been considered for many years to solve the problem of heavy metal pollution and consequently environmental remediation (Vuralli et al., 2017). Biosorbents, like yeasts and algae, possess metal sequestering properties and can affect heavy metal ions concentration. Among the natural materials applied to absorb toxic elements, microbial biomass has attracted increasing attention, owing to their safety for human, tolerating unfavorable circumstances, and being easy to work with. Several methods have been used to decontaminate industrial effluents from heavy metals, yet not all of the approaches were cost-effective or had their finest probable performance (Jakóbik-Kolon et al., 2017).

S. cerevisiae is generally regarded as a safe ideal model organism for studying the mechanism of biosorption, high metal uptake capacity, and high biomass production in low-cost media yeast. It could be obtained from various industrial effluents. There are a great deal of references proving that this yeast can remove heavy metal ions and recover valuable metals. Zrt and Fet family genes are two main classes involved in producing eukaryotic zinc transporters to regulate the intracellular zinc levels in *S. cerevisiae* (Kareena et al., 2021) (Almeida et al., 2020) (Yildiran et al., 2019) (Locatelli et al., 2019).

In addition, single cell protein (SCP) is a general term referring to the protein produced by fungal, bacterial, and yeast biomass which is suitable for human consumption or as animal feeds. It could be utilized as an appropriate protein source to provide essential supplements, such as nitrogen and amino acids for human diets and livestock fodder (Dolatifard & Jafari, 2020; Sharif et al., 2020). The advantages and disadvantages of the SCP depend on the type of microorganisms used for

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[1]*Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran*
[2]*Department of Microbiology, Faculty of Sciences, Saveh Branch, Islamic Azad University, Saveh, Iran*
[3]*Department of Biotechnology, Faculty of Biological Sciences, Tehran North Branch, Islamic Azad University, Tehran, Iran*
*Corresponding author: dr_kumarss_amini@yahoo.com

its production. SCP is produced from inexpensive food grade and waste substrates (whey, fruits residues, hemicelluloses) as carbon and energy sources (Gervasi et al., 2018). In this work, we aimed to isolate *S. cerevisiae* strains from effluent of chemical industries in order to evaluate their capability for biosorption of zinc ions and production of zinc enriched SCP.

2 Material and methods

2.1 Yeast isolation and characterization

In order to isolate *S. cerevisiae* strains, effluent of five alcohol and textile production and fruit processing factories in Tehran (Iran) were collected from the main waste pipes of these areas. The collected waste samples were transferred to the laboratory in ice-cold containers and immediately kept at 4 °C until further examination. The samples were filtered using 0.45 µm pore size filter paper and the filter papers containing waste materials inoculated into the Sabouraud Dextrose Broth (SDB) (Merck, Germany) and incubated in 35 °C for 72 hours. Subsequently, yeast colonies were harvested in YPD medium (2% peptone, 1% yeast extract and 2% glucose) and incubated in 28 °C for 48 hours. Characterization of the *S. cerevisiae* isolates was done based on standard biochemical tests (sugar fermentation method) and ITS and 18S rRNA molecular analysis (Filippis et al., 2017; Okoduwa et al., 2017).

2.2 Molecular identification of isolates

Genomic DNA of *S. cerevisiae* strains was isolated from single colonies using the commercial kit Higher Purity™ Yeast Genomic DNA Extraction (Canvax Biotech, Spain) according to the manufacturer's protocol. The quality and quantity of the extracted DNA were evaluated using spectrophotometry (Nanodrop, Thermofisher, USA) and electrophoresis in 2% agarose gel. To distinguish *S. cerevisiae* from other yeasts, ITS-PCR was employed. The internal transcribed spacers (ITS) (ITS1 and ITS4) regions of the isolates harboring target genes were amplified using specific primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), with reaction conditions as previously described by Suranska et al. (Šuranská et al., 2016). PCR products were sequenced (Bioneer, South, Korea), phylogenic relations of the species were identified using MEGA7 software, and the phylogenic tree was constructed using the neighbor-joining method.

2.3 Detecting the strains carrying Zrt and Fet gene families

In order to isolate the strains harboring Zrt and Fet gene families, multiplex PCR was carried out in a final volume of 50 µL containing 25 µL Master mix (Merck, Germany), 1 µL of template DNA, 25 of DEPC-treated water, and 1 µL of each primer (100 pmol µL−1) (Table 1) (Invitech, Germany) as follows: the initial denaturation step was at 95 °C for 5 minutes, followed by 35 cycles of amplification, including denaturation at 95°C for 1 min, annealing at 54 °C for 1 min, extension at 75 °C for 1 min. The final extension was carried out at 70 °C for 2 minutes. PCR products were electrophoresed in 2% agarose gel in the presence of positive and negative controls; after staining with Redsafe (iNtRon biotechnology, South Korea), they were photographed by gel documentation device.

2.4 Optimization of zinc biosorption and biomass production

The effect of zinc concentration on the efficiency of zinc biosorption as well as biomass production in *S. cerevisiae* strains harboring target genes was investigated. Briefly, various concentrations (0, 10, 25, 50, and 100 µg/mL) of the ZnSO4 solution (Merck, Germany) was added into 500 mL Erlenmeyer flasks containing 200 mL SDB (Sabouraud dextrose broth) culture medium and *S. cerevisiae* pellets (5×10^6 Cell/mL) subjected into the suspension and incubated in 28 °C in the shaking incubator (200 rpm/min) for 72 hours (initial pH = 5.8). The growth rate of the yeasts was evaluated by measuring the optical density (OD) of suspension during the incubation with 24-hour intervals (0 to 72 hrs.) at 600 nm. To assess the biomass production, 10 mL of the suspension was centrifuged at 4000 rpm for 20 min and weight of the sediment was assayed after removing the supernatant. Afterwards, to determine the content of dry biomass, a two-stage drying procedure was employed. The samples were primarily dried in the oven at 60 °C for 2 hours and then heated at 105 °C to reach constant weight. Finally, dried biomass was weighed and reported as cell dry weight (CDW). Total accumulation of

Table 1. Primers used to differentiate and isolate *S. cerevisiae* species harboring target genes.

Name	Primer sequence	bp
Zrt1	F: 5′-AAATGCACTAGAACATGGCG-3′	2102
	R: 5′-TTCATGACTATTTAAATGCCTT-3′	
Zrt2	F: 5'-CGTTCACAATCAGTTCTT-3'	580
	R:5'-CCAACTTCTTCTTCCATTTG-3'	
Zrt3	F: 5'-AACGGGCGTCTCGATAGAAAA-3'	380
	R:5'-AACGGGCGTCCACAAAATCA-3'	
Fet4	F: 5'- GGAGAACTGCCTGTGGAAAA -3'	165
	R:5'-GCCAATGTTCTACCATAGC-3'	
Fet3	F: 5'-CAGTTATACGGTATGAGAGC-3'	210
	R:5'-CTGTAGAGTGACAGTTTGGT-3'	
Fet1	F: 5'-TCAGATATTCAAACTGCAGTACG-3'	250
	R:5'-CTACTTCACCTTTTTCTTCAGA-3'	

zinc in yeast cells was determined by means of atomic absorption spectrophotometry (AAS) (Thermo scientific, USA). Briefly, 1 mg of dry biomass was added to nitric acid and distilled water (1:1) in 25 ml tubes and incubated at 160 °C for 20 min. The obtained biomass was then filtered via filter paper. Finally, AAS method was applied to determine the content of zinc in yeast cell biomass at 213.9 nm wavelength. The experiments were performed in triplicate and were compared to the control.

2.5 Effects of zinc concentrations on target genes expression

To evaluate the effects of zinc on Zrt, Fet, and 18S rRNA (a housekeeping gene to normalize the expression levels of other genes) genes expression, yeast total RNA was isolated by means of EZ-10 Spin Column Fungal RNA Miniprep Kit (Bio Basic, Canada) according to the manufacturer's instructions. cDNA was then synthesized via a AccuPower RocketScript RT PreMix kit (Bioneer, Korea) and quantified using SYBR Green (Life Technologies). Finally, quantitative real time PCR (qRT-PCR) was performed with a RealQ Plus 2x Master Mix Green (Amplicon Co, Denmark) on a thermocycler system (StepOneplus, Thermo Fisher Scientific, USA) in a total volume of 20 µL and an initial incubation at 95 °C for 15 mins, followed by 40 cycles of 15 s at 94 °C and 1 min at 62 °C. Melting curve analysis was employed to validate the specificity of the expected PCR product as well as nonoccurrence of primer-dimer formation. All the reactions were carried out in triplicate and the results were analyzed via threshold cycle (Ct) values.

2.6 Effects of pH values on zinc biosorption and biomass production

The effects of pH values on *S. cerevisiae* strains were studied on the isolate with the maximum growth rate, zinc uptake, and the highest levels of target gene expression. Three sets of experiment were conducted on the selected isolate. The yeast inoculated in a 100-mL SDB medium containing 25 µg/mL zinc ions at different pH values (3, 4, 5, and 6) adjusted by means of 0.1 M NaOH and 0.1 M HCL (IranAzma, Iran). The pH was measured using Hanna Instruments HI 9813-6N pH/EC/TDS Meter (Hanna Instruments, USA) and left for 25 minutes to reach equilibrium. Afterwards, estimation of growth rate and total accumulation of zinc in yeast cells, in addition to determination of target genes expression levels in zinc absorbed yeasts, were performed as mentioned before.

2.7 Total protein content

Total protein content of *S. cerevisiae* strains was assayed in the determined optimum condition. For this purpose, Kjeldahl method was carried out and protein was measured indirectly by conversion of total nitrogen content into total protein content as detailed by Jung et al. (2003).

2.8 Statistical analysis

In the current study, all the measurements were performed in triplicate and the obtained results were the average of the three replications. SPSS software v.16 was utilized for statistical analysis. The data were analyzed using ANOVA. The numerical data were presented as mean±SD (standard deviations) and statistical significance was determined by P values < 0.05.

3 Results and discussion

3.1 Isolated yeasts

A total of 100 samples from industrial effluents were collected. The initial pH of the industrial effluents was about 6.0 and its zinc concertation was about 48.000 µg/L. Totally, 52 strains were isolated from the collected samples. Molecular analysis showed that three out of 52 strains were detected harboring Zrt and Fet genes family. Analysis of ITS gene sequences of white to cream, glabrous, smooth, and yeast-like colonies of isolated strains to determine phylogenetic relationship indicated two isolated microorganisms identified as *S. cerevisiae AUMS 10233* species (Figure 1), which made them appropriate candidates to be assayed in the rest of the investigation. In order to produce yeast biomass as a source of SCP, identifying yeast species with optimal properties is of great value. *S. cerevisiae*, as a biosorbent yeast, has been long studied and its valuable properties have been confirmed. As shown in our study, this yeast is easily cultivated using a cheap medium and is also a by-product in industry wastewater, which can be easily manipulated at the molecular level. It could also be considered as a model system for the accumulation of metals in relatively high concentrations. The use of *S. cerevisiae* yeast is highly frequent in several industries owing to its unique nature despite its average capacity for metal extraction, compared to other fungi (Massoud et al., 2019).

3.2 Effects of zinc concentration on biomass production and zinc biosorption

Microorganism growth under exposure to metal cations of interest was evaluated to gauge the applicability of the species for biosorption. Table 2 depicts the effect of initial zinc concentrations on the *S. cerevisiae* biomass yield and biosorption. The highest growth rate (OD600 = 2.520) and biomass production (0.206 g of dry cells /200 mL SDB medium) was observed at 24 hours after the inoculation in 25 µg/mL of zinc concentration (Figure 2). The results showed that with the increase in zinc concentrations up to 25 µg/mL, the absorptions significantly improved; however, there were no significant changes in higher concentrations. This is in accordance with studies reporting that as the number of zinc ions increased, their absorption to the surface of the yeasts increased. Therefore, higher biosorption would be observed (Feng et al., 2018; Mwandira et al., 2020). The yeast cells grew in all the tested concentrations of zinc cations, showing acceptable resistance to zinc contamination up to 100 µg/mL. The maximum uptake of zinc by yeast cells was observed at 24 h after the inoculation, suggesting that the increment of incubation time after 24 h did not positively affect biosorption. The growth rate increased with the increase in zinc cations and then become constant and saturated with the zinc concentrations higher than 25 µg/mL. Previous studies have shown that at higher concentrations of cations, the lack of available binding sites results in further unabsorbed ions (Kılıç et al., 2014) (Ucun et al., 2009) (Ekmekyapar et al., 2006).

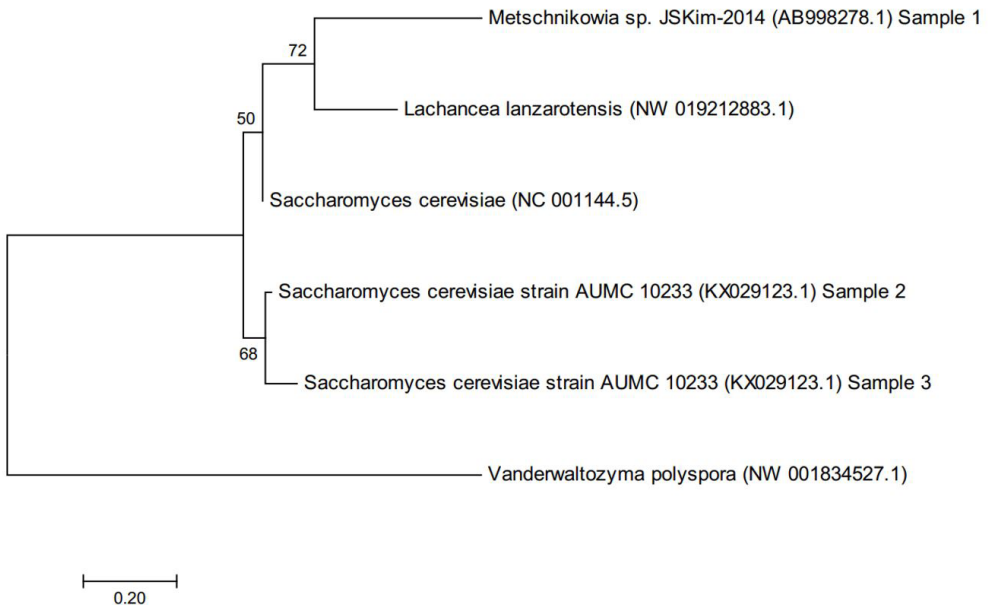

Figure 1. Phylogenetic relationships via a neighbor-joining analysis of 18S rDNA sequences, showing the position of strains (sample 1, 2, and 3) isolated from industrial effluent. Numbers at nodes indicate the levels of bootstrap support based on 1000 replicated datasets.

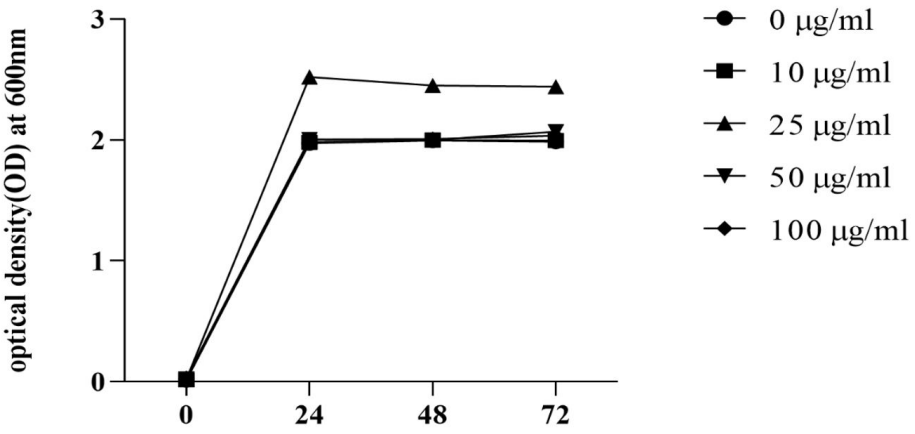

Figure 2. Growth rate of *S. cerevisiae* isolates grown in SDB media after three days at different ZnSO4 concentrations.

3.3 Effects of zinc concentration on target genes expression

The results of qRT-PCR revealed that gene expressions of Zrt1 and Fet4 were significantly upregulated in the optimum zinc concentration (25 µg/mL) and after 24 hours of incubation time compared to the control group (zinc free SDB medium). There were no statistically significant changes in the expression levels other family members. The Zrt1 expression reached a maximum level following 24 h of incubation in 25 µg/mL of zinc concentration; meanwhile, the maximum level of Fet4 expression was observed in 50 µg/mL of zinc concentration (Figure 3). Generally, the high-affinity transporters undergo rapid upregulation in response to starvation for specific nutrients, such as metal ions and glucose. Our observations are consistent with those of previous studies, suggesting that extracellular zinc uptake by *S. cerevisiae*, in severe zinc restriction, occurs using a high affinity zinc transporter, Zrt1 (Lehtovirta-Morley et al., 2017). However, this yeast can, to some extent, use Fet4, as a low-affinity transport protein, for absorbing zinc as well as iron and copper into the cell under conditions where the amount of zinc in the environment is low (but not severe) (Massoud et al., 2019).

3.4 Optimum pH for biomass production and zinc sorption

The maximum growth rate, zinc accumulation, and cell dry weight were observed at pH=6, suggesting the optimum

Table 2. Effects of zinc concentrations on the yield of dry yeast biomass and zinc uptake by *S. cerevisiae*.

$ZnSO_4$ concentrations in SDB medium														Time
100			50			25			10			0		
RSD	Zn^{2+} uptake	CDW	RSD	Zn^{2+} uptake	CDW	RSD	Zn^{2+} uptake	CDW	RSD	Zn^{2+} uptake	CDW	RSD	Zn^{2+} uptake	CDW	
0.4	51.02	0.178	0.4	31.57	0.016	0.6	22.14	0.206	1	12.15	0.114	-	-	0.102	24
0.8	40.39	0.174	0.5	27.05	0.172	0.5	16.58	0.198	0.6	8.71	0.120	-	-	0.110	48
0.3	33.78	0.158	0.4	19.25	0.150	0.5	16.06	0.122	0.5	10.34	0.110	-	-	0.114	72

Time: incubation time after inoculation (h), CDW: cell dry weight (g/200 ml SDB), Zn^{2+} uptake: absorbed zinc by yeast (ppm), RSD: relative standard deviation (%), 0, 10, 25, 50 and 100: different concentrations of $ZnSO_4$ in SDB medium (µg/ml) and 0 means SDB medium without the addition of zinc (as control).

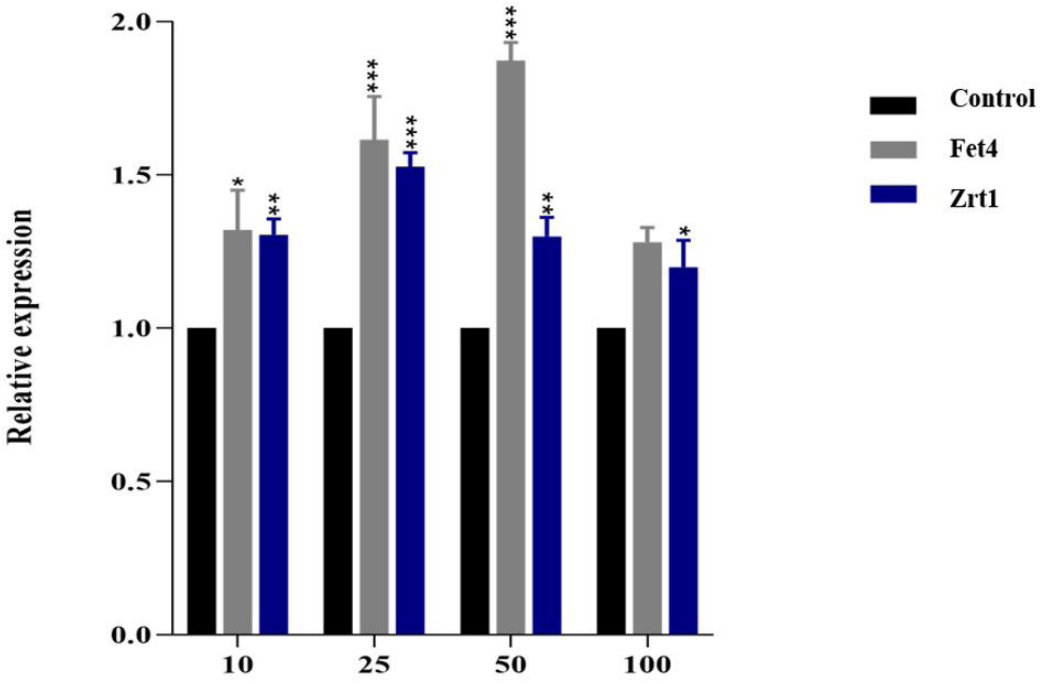

Figure 3. Relative expression levels of Zrt1 and Fet4 genes in *S. cerevisiae* strains grown in the SDB medium with different concentrations of zinc ions and after 24 hours of incubation versus the control (zinc free SDB). The maximum Zrt1 upregulation was observed in 25 µg/mL of zinc concentration. However, Fet4 expression levels were found to significantly increase in the presence of 50 µg/mL of zinc. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Table 3. Effect of initial pH of growth medium on biomass yield.

pH	3	4	5	5.8	6
OD	1.780	1.823	2. 200	2.518	2.556
CDW	0.10	0.176	0.18	0.203	0.218

pH= 5.8: Initial pH of SDB medium, OD: optical density, CDW: cell dry weight (g/200 ml SDB).

pH in 24 incubation and 25 µg/mL of zinc concentration for the maximum biomass production and zinc sorption (Table 3). In addition, the maximum level of Fet4 gene upregulation was observed in pH=4. pH value is one of the most significant environmental factors involved in heavy metal ions sorption as it affects not only the solution chemistry of the heavy metal ions, including redox reactions, hydrolysis, complexation, precipitation, and the speciation, but also the site dissociation of the microorganism biomass. Previous studies have reported that the optimum pH value for different metal ions biosorption is different since the pH of solutions affect ionized groups in the yeast cell wall (Zinicovscaia et al., 2020a) (Figure 4). The effect of pH on the metal ions sorption was investigated in the range of 3 to 9 and the optimal pH values have been reported as 4 for nickel, 5.8 for lead, zinc, and cadmium (Massoud et al., 2020) and 5 to 9 for copper (Zinicovscaia et al., 2020a; Zinicovscaia et al., 2020b). At low pH values, low metal sorption is explained by the competition between H^+ and zinc ions for binding to the sites of the yeast cell wall while increased pH results in an increase in ligands with negative charges for zinc ions. On the other hand, at alkaline pH, the interaction of OH^- groups with zinc positive charge ions leads to the formation of zinc hydroxide and decreasing biosorption capacity (Hadiani et al., 2018; Zinicovscaia et al., 2020c). Our findings are consistent with the results obtained from investigating *Oceanobacillus profundus*, *Pseudevernia furfuracea*, and *S. cerevisiae*, which implied an effective pH range of 2 to 8 (Mwandira et al., 2020) (Kılıç et al., 2014) (Farhan & Khadom, 2015; Wang, 2012).

3.5 Protein content evaluation

The maximum protein content of dried biomass of *S. cerevisiae* cultivated at optimal condition was obtained to be 50.6%, suggesting an acceptable commercial candidate for SCP production. Previous investigations have indicated that *S. cerevisiae* species has large amounts of protein with polysaturated fatty acids and minerals. Hezarjaribi et al. (2016), Liu et al. (2013), and Maragatham & Panneerselvam (2011) achieved respectively 44.6%, 46.09%, and

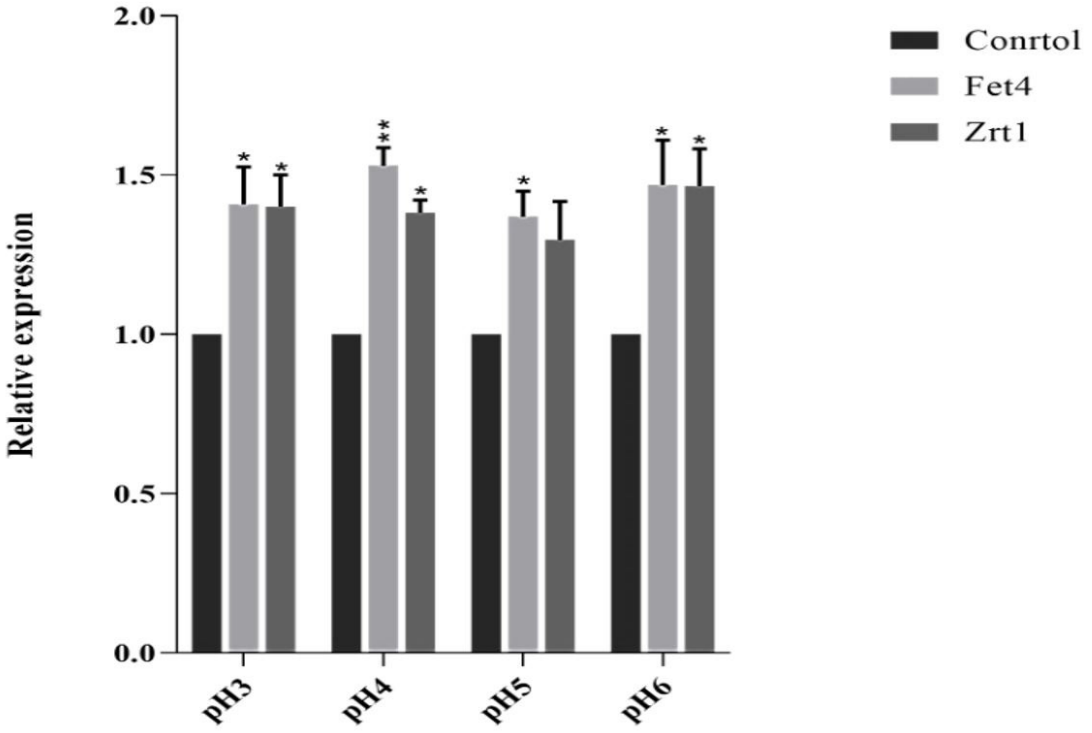

Figure 4. Relative expression levels of Zrt1 and Fet4 genes in different pH values versus the control (initial pH:5.8). The maximum Fet4 upregulation level was observed in pH=4. *P < 0.05.

34% protein in dried cell biomass of *S. cerevisiae* using optimal culture composition and condition.

4 Conclusion

The selection of an appropriate culture medium, the choice of capable yeast strain, as well as an optimal condition are pivotal factors in efficient biomass and production and metal biosorption. The results of the current investigation revealed that *S. cerevisiae* species isolated from industrial effluent could be applied for zinc biosorption. In addition, these species biomass are potentially available for production of zinc enriched single cell protein and this way, it would be possible to kill two birds with one stone. Our study revealed the possibility of upgrading low value industrial effluents to SCP with these highly promising yeasts, which could be utilized as a high-quality feedstock.

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