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Abstract: An antibiotic produced by strain Streptomyces parvulus showing activity against Staphylococcus citreus was subjected to various optimization parameters for enhancing its production. Nutritional and physiological parameters produced by S. parvulus under shaken flask conditions were determined. Optimization of these parameters led to 11% increase in antibiotic activity with a mean zone of inhibition of 42 mm.
Highest antibiotic production was obtained at 250 rpm for 14 days with optimum temperature of 28oC and pH 7. Kuster's modified medium containing glycerol 0.7% (v/v), casein 0.03% (w/v), NaCl 0% (w/v), phosphate 0.25% (w/v), KNO3 0.1% (w/v) and CaCO3 0.0015% (w/v) concentration was found ideal.

Title: Optimization of an anti *Staphylococcus* antibiotic produced by tropical soil dwelling *Streptomyces parvulus*

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ABSTRACT

An antibiotic produced by strain *Streptomyces parvulus* showing activity against *Staphylococcus citreus* was subjected to various optimization parameters for enhancing its production. Nutritional and physiological parameters produced by *S. parvulus* under shaken flask conditions were

determined. Optimization of these parameters led to 11% increase in antibiotic activity with a mean zone of inhibition of 42 mm. Highest antibiotic production was obtained at 250 rpm for 14 days with optimum temperature of 28^{o}C and pH 7. Kuster's modified medium containing glycerol 0.7% (v/v), casein 0.03% (w/v), NaCl 0% (w/v), phosphate 0.25% (w/v), KNO_3 0.1% (w/v) and $CaCO_3$ 0.0015% (w/v) concentration was found ideal.

Key words: *Streptomyces parvulus*; *Staphylococcus citreus*; Bioassay

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1. Introduction

Novel antibiotics are continuously in demand due to the inevitable rise of antibiotic-resistant strains of pathogenic bacteria, reducing morbidity and mortality of life's expectancy (Fischbach and Walsh 2009). Among the pathogenic bacteria *Staphylococcus citreus* needs a special mention on account of its pathogenicity.

S. citreus, a virulent strain has risen due to spontaneous split off of pure line strains of pathogenic *S. aureus* (Pinner and Voldrich 1932) which causes skin infections, pneumonia, meningitis, endocarditis, toxic shock syndrome, septicemia and arthritis (Dilsen et al. 1961; Chambers 2001).

Research done to harness potential drug candidates against this pathogen has not been reported, although concerted efforts to harness potential drugs against multi drug resistant strains of *Staphylococcus aureus* such as MRSA is underway (Demain and Sanchez 2009).

Actinobacteria have proven to be prolific producers of secondary metabolites among all microbial organisms, accounting for 45% of all microbial metabolites of which 80% (7,600 compounds) are produced by genus *Streptomyces* (Berdy 2005).

According to Watve et al. (2001) predictive modeling of genus *Streptomyces* suggests that over 150,000 bioactive metabolites from this genus still needs to be discovered.

Antibiotic biosynthesis in *Streptomycetes* has been reported to be highly dependent on the nutritional and physiological factors prevailing during its growth as it helps in cell proliferation which expresses genetic information favoring secondary metabolism (Abbanat et al. 1999).These metabolic processes are species specific and can be enhanced or minimized under different physiological conditions (Yarbrough et al. 1992; Abbanat et al. 1999; Selvin et al. 2009; Visalakchi and Muthumary 2009; Rezuanul et al. 2009; Elleuch et al. 2010; Nanjwade et al. 2010; Panda et al. 2011; Chhabra and Keasling 2011; Darabpour et al. 2012; Mangamuri et al. 2012; Singh and Rai 2012; Luthra and Dubey 2012; Gunda and Charya 2013). Thus, it is essential to standardize growth conditions of the producer strain, for maximum synthesis of its bioactive molecules (Sujatha et al. 2005; Olmos et al. 2013).

Classical strain improvement despite being laborious and time consuming is still widely used due to its high success rate behind improved production titers of antibiotics such as penicillin, cephalosporin C, tylosin, salinomycin, chlortetracycline and tetracycline (Chhabra and Keasling 2011).

In the course of screening actinobacteria for antibiotic compounds, a strain identified as *Streptomyces parvulus* showed broad spectrum activity as revealed by perpendicular streak (Badji et al. 2007) and agar well diffusion method (Devillers et al. 1989). The present communication deals with the optimal parameters required for maximizing antibiotic

production by *S. parvulus* against pathogenic bacteria *Staphylococcus citreus*: We claim this to be the first such report.

2. Methods

2.1. Location and sampling

The actinobacterial strain CFA-9, deposited at Goa University Fungal Culture Collection (GUFCC 20101) was isolated from forest soil, Canacona, Goa, India (latitude 14°59'45.76"N and longitude 74°03'02.17"E). Isolation of this strain was carried out using a novel baiting technique, which employs a microcosm with Arginine Vitamin Agar (AVA) medium coated slides to specifically capture *ex situ* actinobacterial diversity (Velho-Pereira and Kamat 2011; 2012).

2.2. Taxonomic and molecular identification of the producer organism

The morphological and cultural characteristics of the strain CFA-9 were studied by using traditional criteria of classification (Locci 1989; Cross and Goodfellow 1973). The micromorphological studies were done using light and scanning electron microscopy (SEM) (Williams and Davies 1967).

For molecular identification, genomic DNA of the strain was extracted and its quality was evaluated as a single distinct band on 1.2%

agarose gel. The fragment 16S rRNA gene was amplified by PCR and the amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic analyser. Consensus sequence of 1347bp 16S rRNA gene was generated from forward and reverse sequence data using aligner software. This work was done at Xcelris Labs Ltd. (www.xcelerislabs.com). The sequence has been deposited in Genebank database (http://www.ncbi.nlm.nih.gov/genbank/submit).

Phylogenetic analyses were conducted using MEGA v5 (Tamura et al. 2011). The 16S rRNA gene sequence of strain CFA-9 was aligned using the Clustal W program against corresponding nucleotide sequences of representatives of *Streptomyces* genus retrieved from Genbank (http://www.ncbi.nlm.nih.gov/genbank). Phylogenetic tree was inferred by the maximum-likelihood method (Felsenstein 1985) based on the Hasegawa–Kishino–Yano (HKY) (Hasegawa et al. 1985) model. Tree topologies were evaluated by bootstrap analysis (Felsenstein 1985) based on 1000 resamplings.

2.3. Antibiotic production medium

Kuster's broth (Kuster and William 1964) composed of glycerol (0.8% v/v), Casein (0.03% w/v), NaCl (2% w/v), KNO_3 (0.2 w/v), K_2HPO_4

(0.2% w/v), $MgSO_4.7H_2O$ (0.005% w/v), $CaCO_3$ (0.001% w/v), pH 7 was used as the basic fermentation medium. It was inoculated with a seven day old culture (5% inoculum) under sterile conditions. The culture flasks were fixed on a rotary shaker (Orbitek^R, Scigenics Biotech, Pvt. Ltd., India) 250 rpm; rotation diameter: 2.0 cm, placed in a thermostated cabinet at 28^{o}C for fourteen days fermentation process. All these parameters formed the positive control of the experiment. The chemicals were procured from HiMedia, Mumbai, India.

2.4. Antibiotic bioassay and test organism

Agar well diffusion method (Devillers et al. 1989) was used for detection of antimicrobial activity. Antibiotic bioassay was carried out using the cell free centrifugate (CFC) to detect extracellular production of bioactive metabolites. Uninoculated Kuster's broth was used as a negative control.

The culture broth was subjected to centrifugation at 4000 g for 20 min to obtain a CFC. Medium for bioassay was Muller Hinton agar (Himedia veg, Mumbai, India). Three cores of 6 mm diameter were excised from the Mueller Hinton agar plates, pre-seeded with the test organism using sterile swabs. The wells were filled with the supernatant (50-70 µl) using Accupipet model T1000 (Tarsons Products Pvt. Ltd., Kolkata, India).The

176 plates were incubated at 28°C for 48h and inhibition zones (ZOI) were
177 visualized and measured in millimeters.
178 Since preliminary screening showed highest activity against
179 *Staphylococcus citreus*, the same was chosen as a test pathogenic strain
180 for optimizing all parameters. The test organism *S. citreus* was procured
181 from Department of Microbiology, Goa Medical College, Goa and
182 maintained on nutrient agar media (Himedia, Mumbai, India).
183
184 *2.5. Optimization parameters*
185
186 Antibiotic production was optimized by using different
187 physiological and nutritional parameters *viz*., days of incubation,
188 temperature, pH, sodium chloride, organic and inorganic carbon and
189 nitrogen sources and phosphate. The optimum conditions identified for
190 one parameter were used for optimizing the other parameters sequentially.
191 The efficiency of the optimized parameters was established on the basis of
192 zone of inhibition (ZOI) in mm. All experiments were performed in
193 triplicates (n=3).
194
195 *2.5.1. Days of incubation*
196
197 To study the effect of incubation period on antibiotic production,10
198 ml aliquot of the culture broth was collected aseptically at regular

intervals of 4, 7, 12, 14, 16, 18 and 20th days and the CFC was subjected to bioassay.

2.5.2. Temperature

The optimum temperature for antibiotic production was assayed by incubating the production medium at 25, 28, 32 and 37^{o}C. The control was maintained at 28^{o}C.

2.5.3. pH

Influence of pH on antibiotic production of the strain was determined by adjusting the pH of production medium ranging from 3-11 with 0.1 N NaOH/0.1 N HCl. pH 7 was used as the control.

2.5.4. NaCl concentration (ppm)

The effect of sodium chloride on antibiotic production was studied using different salinity concentrations of 0; 10,000; 15,000; 20,000; 25,000; 30,000 and 35,000 ppm. NaCl concentration of 20,000 ppm was kept as control.

223 *2.5.5. Organic Carbon and Nitrogen source (%)*

224

225 To study the influence of carbon and nitrogen source on antibiotic
226 production, varied concentrations of the respective sources were tested.
227 Glycerol being the sole organic carbon source of the Kuster's broth
228 medium was studied using its varied concentration of 0.5, 0.6, 0.7, 0.8,
229 0.9, 1, 1.2 and 1.3% (v/v). Glycerol concentration of 0.8% (v/v) was kept
230 as control. Casein being the sole organic nitrogen source of the medium
231 was studied using its varied concentration of 0.01-0.09% (w/v). Casein
232 concentration of 0.03% (w/v) was kept as control.

233

234 *2.5.6. Phosphate concentration (%)*

235

236 To study the effect of phosphate mineral (K_2HPO_4) on antibiotic
237 production different concentrations, 0.00029; 0.00057; 0.00086; 0.0011;
238 0.0014; 0.0017; 0.002; 0.0023; 0.0026 and 0.0029% (w/v) were tested.
239 Phosphate concentration of 0.0011% (w/v) was kept as control.

240

241 *2.5.7. Inorganic nitrogen and carbon source (%)*

242

243 The influence of inorganic nitrogen (KNO_3) and carbon source
244 ($CaCO_3$) present in the antibiotic production medium was studied using
245 varied KNO_3 concentration of 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5

and 0.55% (w/v). KNO_3 concentration of 0.1% (w/v) was kept as control and eight varied concentration of $CaCO_3$ i.e. 0, 0.0005, 0.001, 0.0015, 0.002, 0.0025, 0.003, 0.0035% (w/v) was studied. $CaCO_3$ concentration of 0.001% (w/v) was kept as control.

2.6. Statistical Analysis

Results are expressed as a mean of three experiments ± standard deviations (SDs). Statistical analyses were performed using WASP (Web Based Agricultural Statistics Software Package 1.0, (http://www.icargoa.res.in/wasp/index.php) and differences were considered significant if $p \leq 0.05$.

3. Results

3.1. Selection and molecular identification of the strain

Among the five strains isolated from the forest soil, CFA-9 with broad spectrum antimicrobial activity of 66.7% against human pathogens namely Gram negative bacteria *Shigella flexneri*, *Enterobacter aerogens* and Gram positive bacteria *Bacillus subtilis*, *Staphylococcus typhi* and *S. citreus* was selected. Highest zone of inhibition (31 mm) was observed against *S. citreus*.

The strain exhibited grey aerial mycelium, with spiral spore chains and produced a bright yellow pigment and was identified as *Streptomyces parvulus*, with gene bank accession number of KC904376 (Fig. 1).

3.2. Optimization parameters

3.2.1. Days of incubation

The maximum antibiotic production resulting in a mean ZOI of 35 mm was recorded over a period of 14 days. The activity was observed from the 4^{th} day of incubation and reached a maximum on the 14^{th} day showing significant variation ($p \leq 0.05$). Thereafter, with the increase in incubation period, the antibiotic production decreased (Fig. 2).

3.2.2. Temperature

As seen in the Fig. 3, incremental temperature rise led to an increase in the antibiotic production till it reached the optimum, further increase in temperature was accompanied by a decrease in the antibiotic production. Maximum yield of bioactive metabolites was observed when the strain was cultured at optimum temperature of $28^{o}C$ with a mean ZOI of 37 mm which was significant ($p \leq 0.05$). $25^{o}C$ and $32^{o}C$ also showed an

appreciable mean ZOI of 29 and 32 mm respectively. The lowest mean ZOI of 24 mm was observed at 37°C (Fig. 3).

3.2.3. pH

Maximum antibiotic activity occurred at pH 7.0, exhibiting mean ZOI of 37 mm which was significant ($p \leq 0.05$). No antibiotic production was observed at pH 3, 4 and 11. Increasing the pH value led to an increase in the antibiotic production up to certain threshold limit and further increase in values resulted in decrease in the antibiotic production (Fig. 4).

3.2.4. NaCl concentration

Maximum antibiotic production in terms of mean ZOI of 37 mm, was obtained without NaCl and was significant ($p \leq 0.05$). A significant difference was found over control (20,000 ppm concentration) exhibiting a mean ZOI of 33 mm. However activity was observed at all concentration of NaCl (Fig. 5).

3.2.4. Organic Carbon and Nitrogen concentration (%)

The onset and intensity of secondary metabolism is dependent on various nutritional factors like carbon and nitrogen sources.

315 Maximum antibiotic activity was observed with 0.7% glycerol
316 concentration showing a mean ZOI of 35 mm and was significant ($p \leq$
317 0.05). Data indicated that increasing concentration of glycerol from 0.5-
318 0.7% led to an increase in the antibiotic production, thereafter further
319 increase in values from 0.8-1.2% resulted in its decrease (Fig. 6).
320 Maximum antibiotic activity was observed with 0.03% casein
321 concentration showing a mean ZOI of 39 mm and was significant ($p \leq$
322 0.05). However, other concentration of casein also favoured the
323 production of antibiotic compounds (Fig. 7).
324
325 *3.2.5. Phosphate concentration (%)*
326
327 As seen in Fig. 9, 0.25% (w/v) concentration of phosphate resulted
328 in maximum yield of antibiotic exhibiting a mean ZOI of 35 mm and was
329 significant ($p \leq 0.05$). The results also indicated other concentrations of
330 phosphate exhibiting an appreciable ZOI (Fig. 8).
331
332 *3.2.6. Inorganic Nitrogen and Carbon concentration (%)*
333
334 Maximum antibiotic activity was observed with its 0.1% KNO_3
335 concentration exhibiting a mean ZOI of 36 mm which was much higher to
336 that of control (0.2%) with a mean ZOI of 29 mm and was significant ($p \leq$
337 0.05) (Fig. 9).

338 Maximum antibiotic activity was observed with 0.0015% and 0.002%
339 $CaCO_3$ concentration with a mean ZOI of 37 mm in both cases and was
340 significant ($p \leq 0.05$) (Fig. 10).
341
342 **4. Discussion**
343
344 A broad-spectrum antibiotic producing strain *Streptomyces parvulus*
345 exhibiting maximum activity against human pathogenic bacteria
346 *Staphylococcus citreus* was subjected to various optimization parameters.
347 The ability of actinobacterial cultures to produce antibiotics is not
348 fixed and arises from intracellular intermediates through defined
349 biochemical pathways. It can either be greatly increased or completely
350 lost depending on the conditions in which they are grown (Kavitha and
351 Vijayalakshmi 2009). The production of most antibiotics is regulated by
352 complex biosynthetic pathways encoded by physically clustered genes
353 (Sevcikova and Kormanec 2004).
354 *S. parvulus* have been reported to produce antibiotics like
355 Actinocin, Actinomycin, Borrelidin, Hydroxyectoine and Manumycin
356 (StreptomeDB, www.pharmaceuticalbioinformatics.de/streptomedb).
357 Studies dealing with optimizing such compounds are scarce with the
358 exception of Actinomycin (Foster and Katz 1981; Sousa et al. 2001).

359 However, studies by Genilloud et al. (2011) have shown that inspite of
360 taxonomic relatedness of these strains, the conditions for antibiotic
361 production were strain dependent.

362 Period of incubation had a profound effect on antibiotic production
363 with maximum zone of inhibition exhibited after 14 days. Further increase
364 in incubation period led to decrease in the antibiotic production which is
365 in accordance to the previous studies reporting that antibiotic production
366 usually occurs in late exponential and stationary phase (El-Nasser et al.
367 2010; Singh and Rai 2012).

368 Maximum antibiotic production at 28°C was in agreement to the
369 previous reports indicating that optimal temperature for antibiotic
370 production is usually in the range of 26°C to 35°C exhibited by several
371 *Streptomyces* species (Elliah et al. 2004; Rizk et al. 2007; Mustafa 2009;
372 Ghosh and Prasad 2010; Elleuch et al. 2010; Atta et al. 2011; Mangamuri
373 et al. 2012; Singh and Rai 2012; Vijayakumar et al. 2012; Gunda and
374 Charya 2013).

375 Antibiotic production in the present study was affected by change in
376 pH of growth medium which is a significant factor affecting nutrient
377 solubility and uptake, enzyme activity, cell membrane morphology by
378 product formation and oxidative reduction reactions (Bajaj et al. 2009;
379 Vijayabharathi et al. 2012). This study found pH 7 as the optimum for
380 antibiotic production and decrease or increase in these values led to
381 complete loss of antibiotic production. Thus the data, is in accordance to

382 the previous reports illustrating pH 7 as optimal for enhancing antibiotic
383 production, exhibited by most *Streptomyces* sp. strains (Elleuch et al.
384 2010; Singh and Rai 2012; Gunda and Charya 2013). Besides, *S.*
385 *coelicolar* (Bystrykh et al. 1996), *S. hygroscopicus* D1.5 (Bhattacharya et
386 al. 1998), *S. torulosus* KH-4 (Atta et al. 2010), *S. viridodiastaticus* (El-
387 Nasser et al. 2010), *Streptomyces cheonanensis* (Mangamuri et al. 2012)
388 also stated maximum activity at pH 7. This phenomenon could be
389 attributed to the adaptation of the strain to alkaline soils from which it
390 has been isolated (Rezuanul et al. 2009).
391 Sources like NaCl had no significant effect on the antibiotic
392 production and were consistent with previous report with respect to
393 neomycin production (Kavitha and Vijayalakshmi 2009).
394 Antibiotic synthesis is highly dependent on utilization of the
395 preferred carbon sources. The results of this study revealed that maximum
396 antibiotic activity was observed with 0.7% (v/v) glycerol concentration.
397 Glycerol as the better carbon source for enhancing antibiotic production
398 by *Streptomyces* has been reported in the previous studies (Selvin et al.
399 2009; Elleuch et al. 2010; Singh and Rai 2012; da Silva 2012). According
400 to Shikura et al (2002), when glycerol is used as the precursor, it forms a
401 β-ketoacyl-CoA, a process similar to polyketide biosynthesis where a
402 dihydroxyacetone-type-C_3 unit is derived from glycerol to create a β-keto
403 ester leading to a γ-butyrolactone autoregulators which is regarded as

404 *Streptomyces* hormones that trigger the onset of secondary metabolism in
405 general and that of antibiotic production in particular.

406 Other than the carbon, assimilation of nitrogen source is also
407 crucial for antibiotic production and is regulated by complex mechanisms
408 of glutamate synthetases (Rodríguez-García et al. 2009, Kavitha and
409 Vijayalakshmi 2009; Selvin et al. 2009; Saha et al. 2010; Vijayabharathi
410 et al. 2012; da Silva 2012). Our study revealed 0.03% casein
411 concentration as the optimal for maximum antibiotic production.

412 Phosphate is also a major factor in antibiotic biosynthesis and
413 expression of phosphate-regulated genes in *Streptomyces* species is
414 modulated by the two-component system PhoR-PhoP (Martin and Demain
415 1980; Rodríguez-García et al. 2009). Dipotassium hydrogen phosphate
416 (K_2HPO_4) is being reported as the most favourable salt for its production.
417 Our results showed 0.25% (w/v) phosphate as optimal for antibiotic
418 production. This data corroborates with the findings of Harold 1966;
419 Kishimoto et al. 1996; Kavitha and Vijayalakshmi 2009; El-Nasser et al.
420 2010; Mangamuri et al. 2012.

421 Among the inorganic carbon and nitrogen sources, maximum
422 antibiotic production was obtained with 0.0015% and 0.002% (w/v)
423 concentration of $CaCO_3$ and 0.1% (w/v) concentration of potassium
424 nitrate. $CaCO_3$ being used as a source of Ca^{+2} enhances antibiotic
425 production and also aids in maintaining the pH of the medium (Hamedi et
426 al. 2004; Basavaraj et al. 2011). Potassium nitrate as superior to other

427 inorganic nitrogen sources has been reported (El-Nasser et al. 2010) and
428 present findings confirms the same.

429 This study identified a set of optimizing parameters such as a
430 period of 14 days of incubation,with optimum temperature of 28^{O}C and
431 pH of 7 and Kuster's modified medium containing glycerol 0.7% (w/v),
432 casein 0.03% (w/v), NaCl 0% (w/v), phosphate 0.25% (w/v), KNO_3 0.1%
433 (w/v) and $CaCO_3$ 0.0015% (w/v) concentration, that culminated in 11%
434 higher yield of antibiotic production with a mean ZOI of 42 mm against
435 clinical pathogenic strain, *Staphylococcus citreus*. It also highlights the
436 need to screen tropical soil actinobacteria against such rising harmful
437 human pathogen and obtain potential antibiotics that could also serve as
438 targets to MRSA like pathogens.

439

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445

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Legends for Figures

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequence showing the relations between antibiotic producer strain *Streptomyces* strain CFA-9 and type species of the genus *Streptomyces* within the order actinomycetales under actinobacteria. The numbers at the nodes indicate the levels of bootstrap support based on maximum-likelihood analyses of 1000 resampled data sets (only values >50% are shown). The scale bar indicates 0.002 substitutions per nucleotide positions.

Fig. 2. Effect of incubation periods on antibiotic production. Bars indicate standard deviation of the mean and the superscripts indicate significant differences ($p \leq 0.05$).

Fig. 3. Effect of temperature on antibiotic production. Bars indicate standard deviation of the mean and the superscripts indicate significant differences ($p \leq 0.05$).

Fig. 4. Effect of pH on antibiotic production. Bars indicate standard deviation of the mean and the superscripts indicate significant differences ($p \leq 0.05$).

721 Fig. 5. Effect of NaCl (ppm) on antibiotic production. Bars indicate
722 standard deviation of the mean and the superscripts indicate significant
723 differences ($p \leq 0.05$).
724
725 Fig. 6. Effect of different concentrations of glycerol (%) as organic
726 carbon source on antibiotic production. Bars indicate standard deviation
727 of the mean and the superscripts indicate significant differences ($p \leq$
728 0.05).
729
730 Fig. 7. Effect of different concentrations of casein (%) as organic nitrogen
731 source on antibiotic production. Bars indicate standard deviation of the
732 mean and the superscripts indicate significant differences ($p \leq 0.05$).
733
734 Fig. 8. Effect of phosphate (%) on antibiotic production. Bars indicate
735 standard deviation of the mean and the superscripts indicate significant
736 differences ($p \leq 0.05$).
737
738 Fig. 9. Effect of different concentrations of KNO_3 (%) as inorganic
739 nitrogen source on antibiotic production. Bars indicate standard deviation
740 of the mean and the superscripts indicate significant differences ($p \leq$
741 0.05).
742

743 Fig. 10. Effect of different concentrations of $CaCO_3$ (%) as inorganic
744 carbon source on antibiotic production. Bars indicate standard deviation
745 of the mean and the superscripts indicate significant differences ($p \leq$
746 0.05).
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Figure

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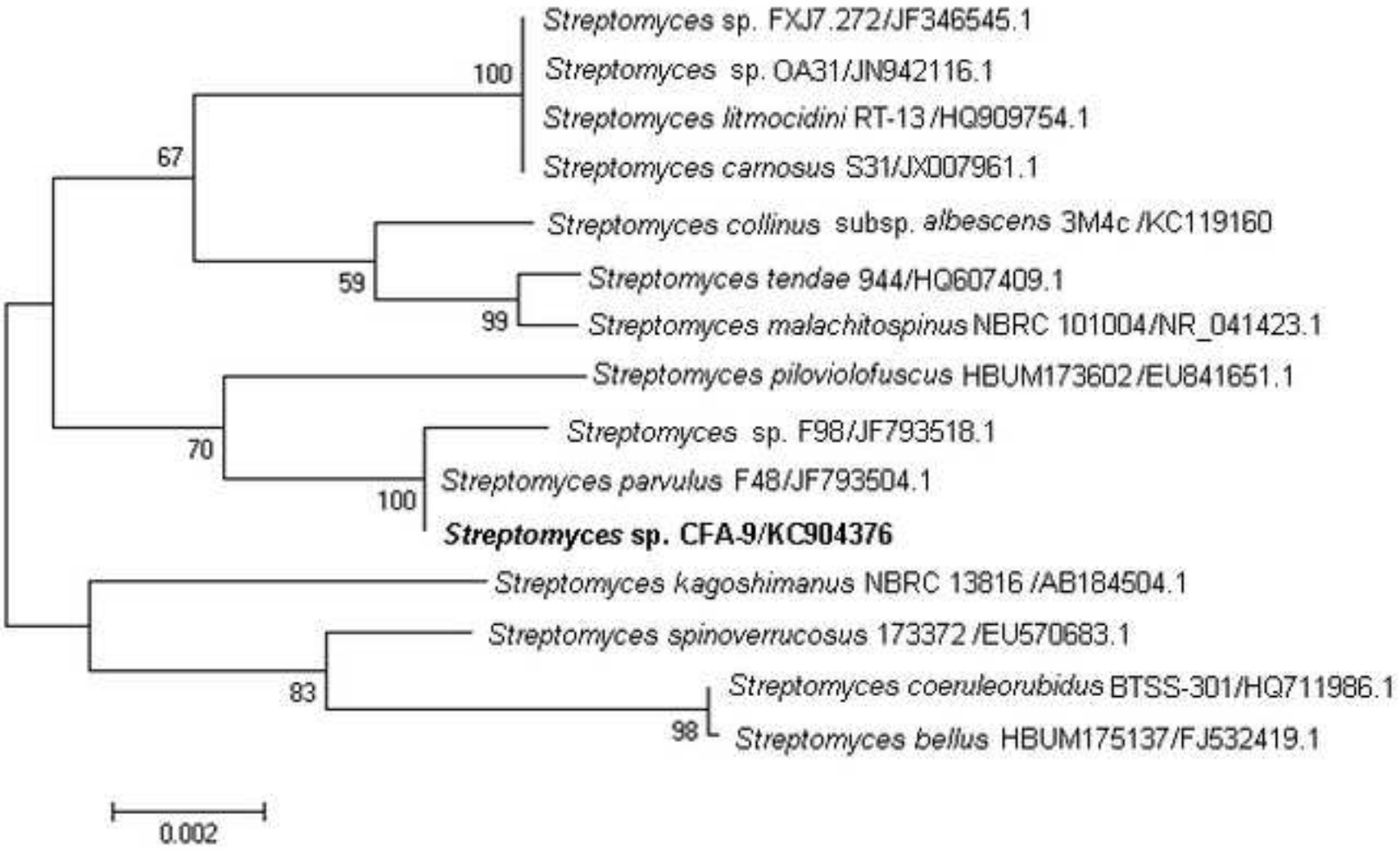

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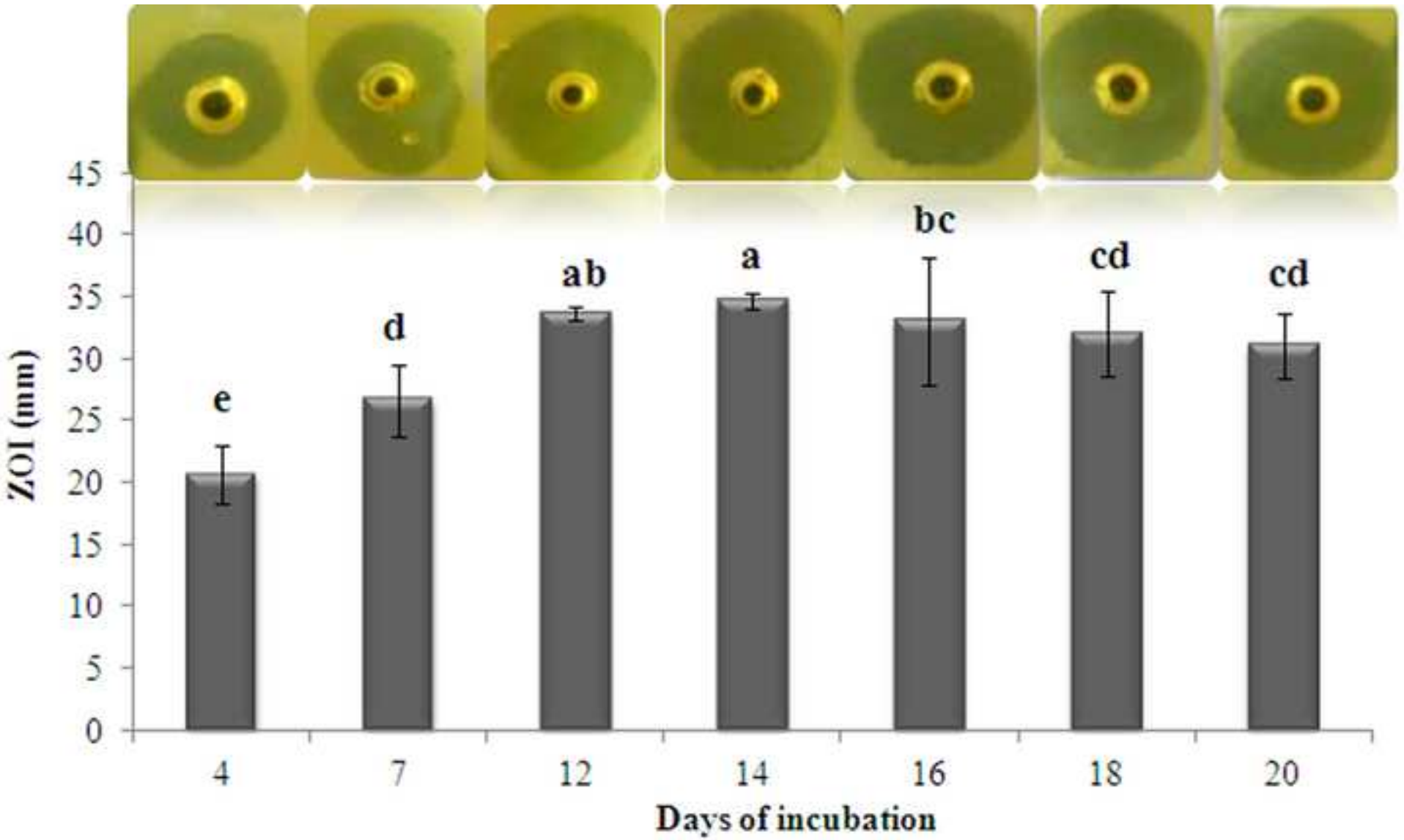

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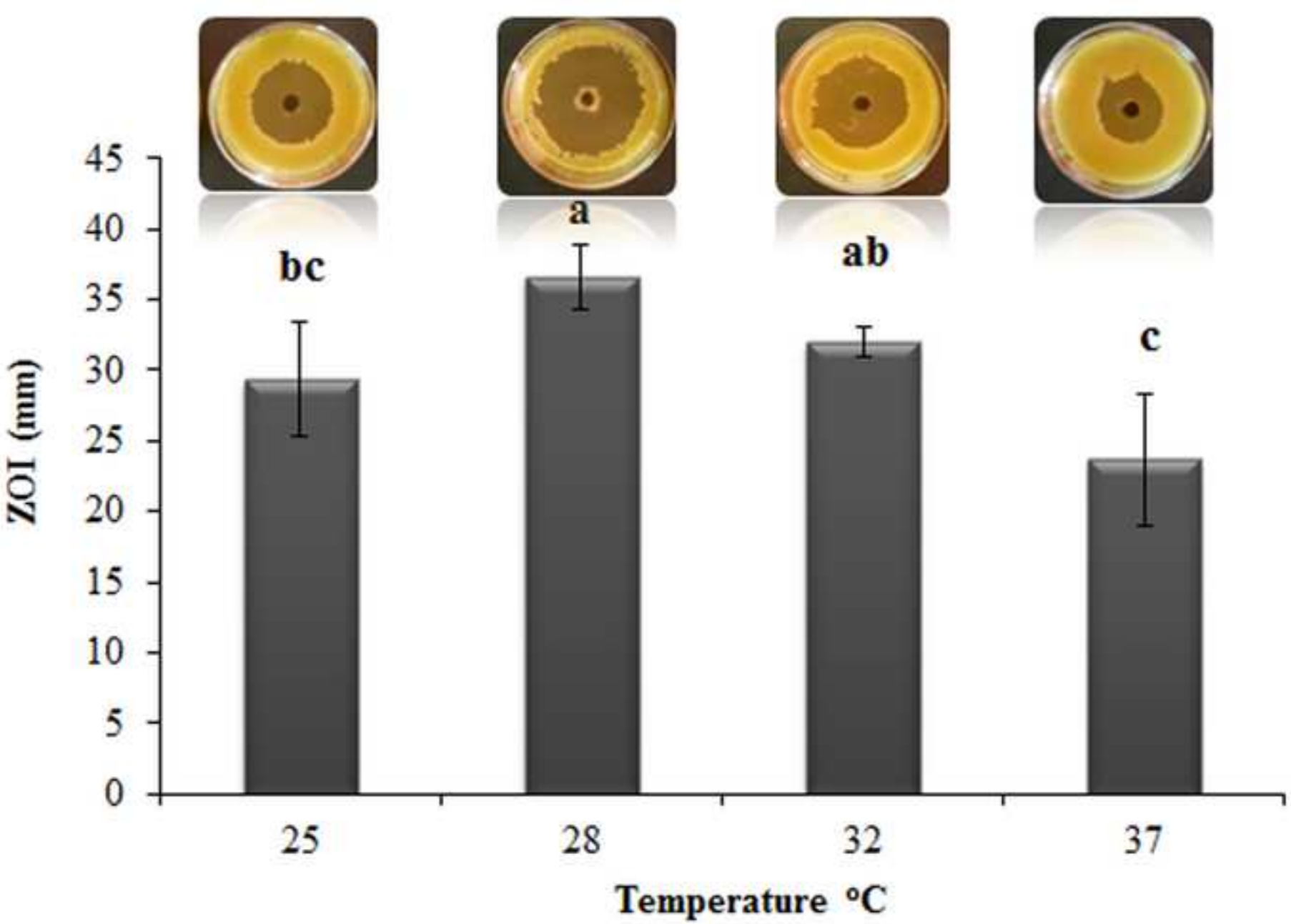

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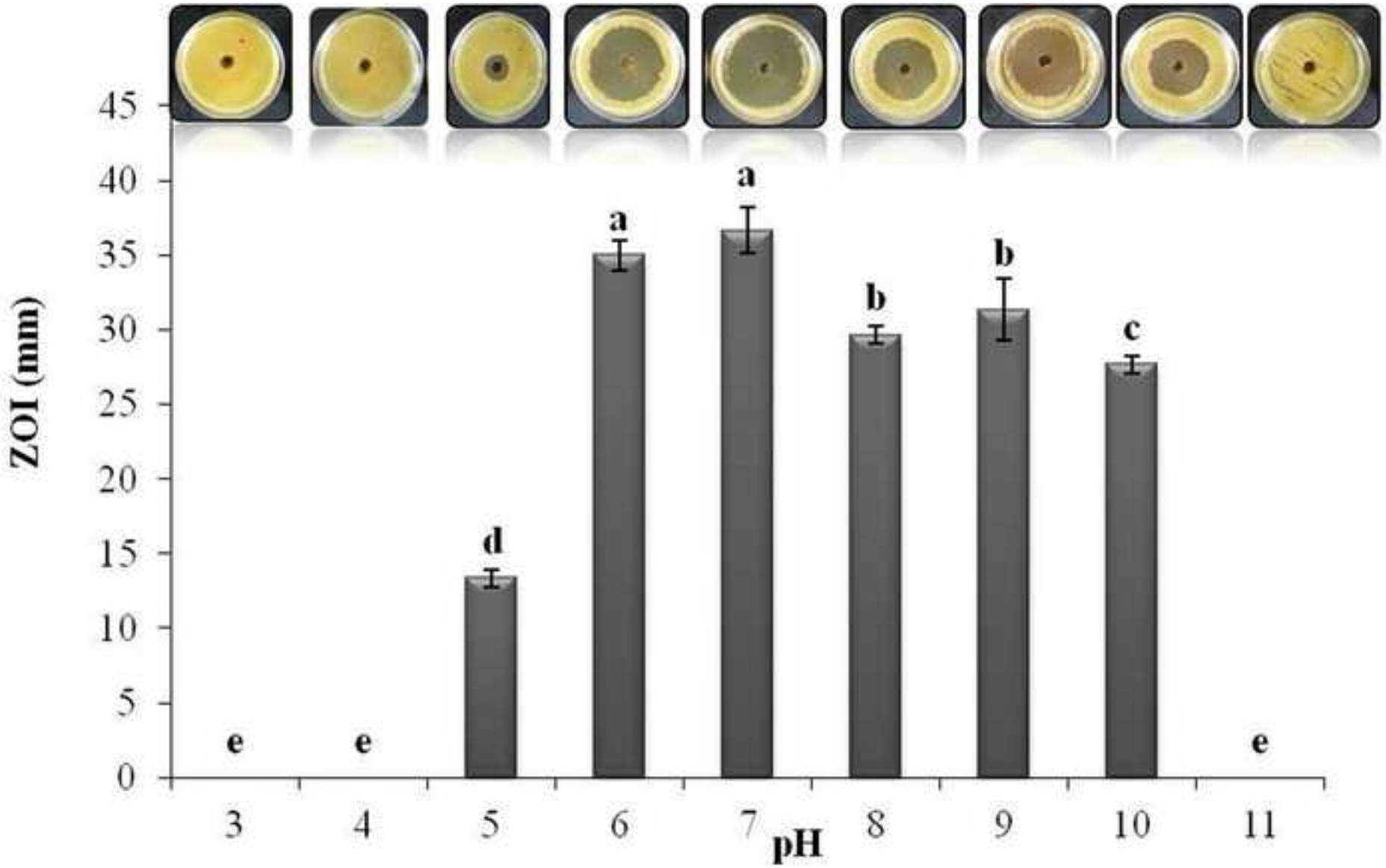

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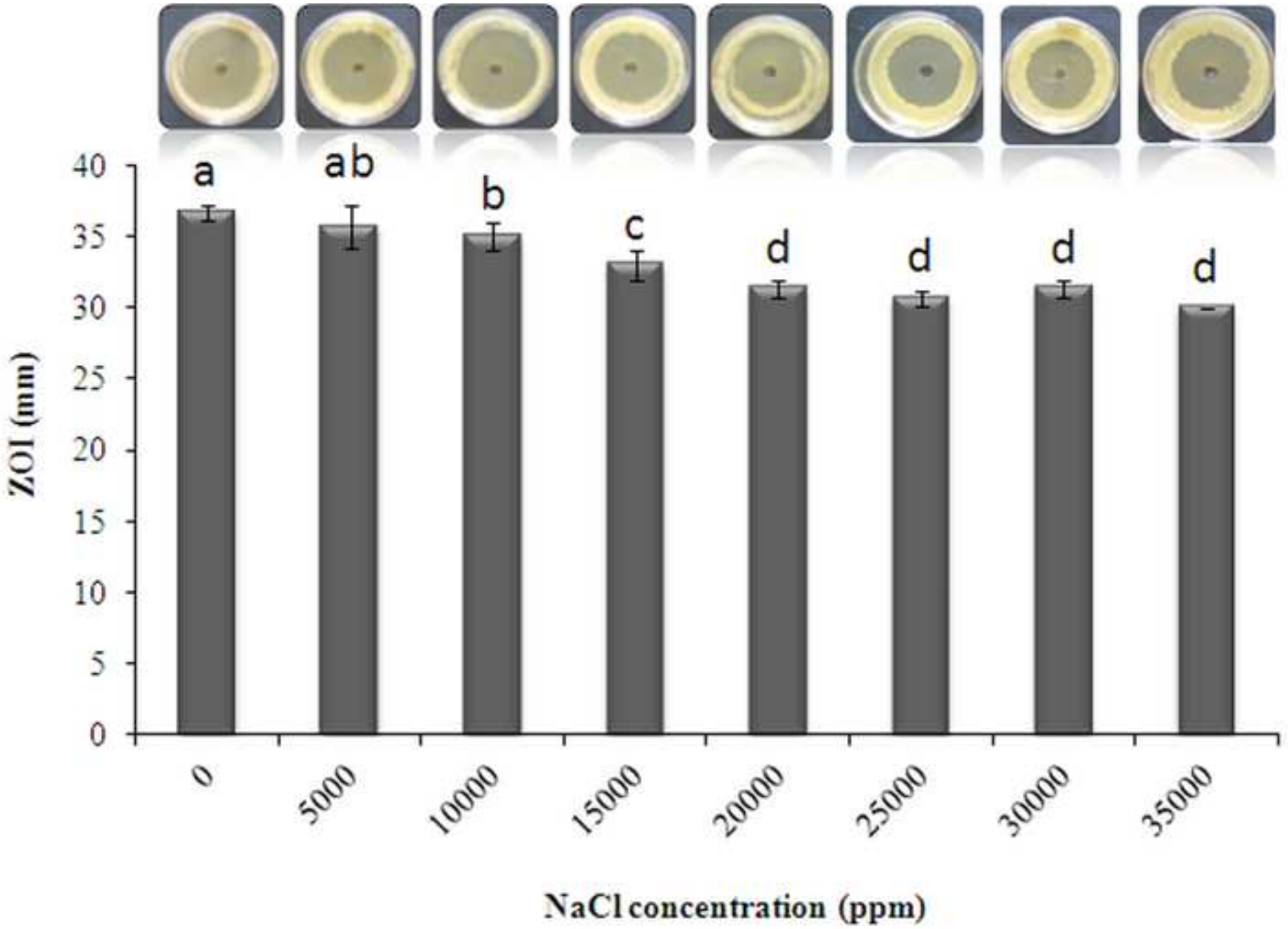

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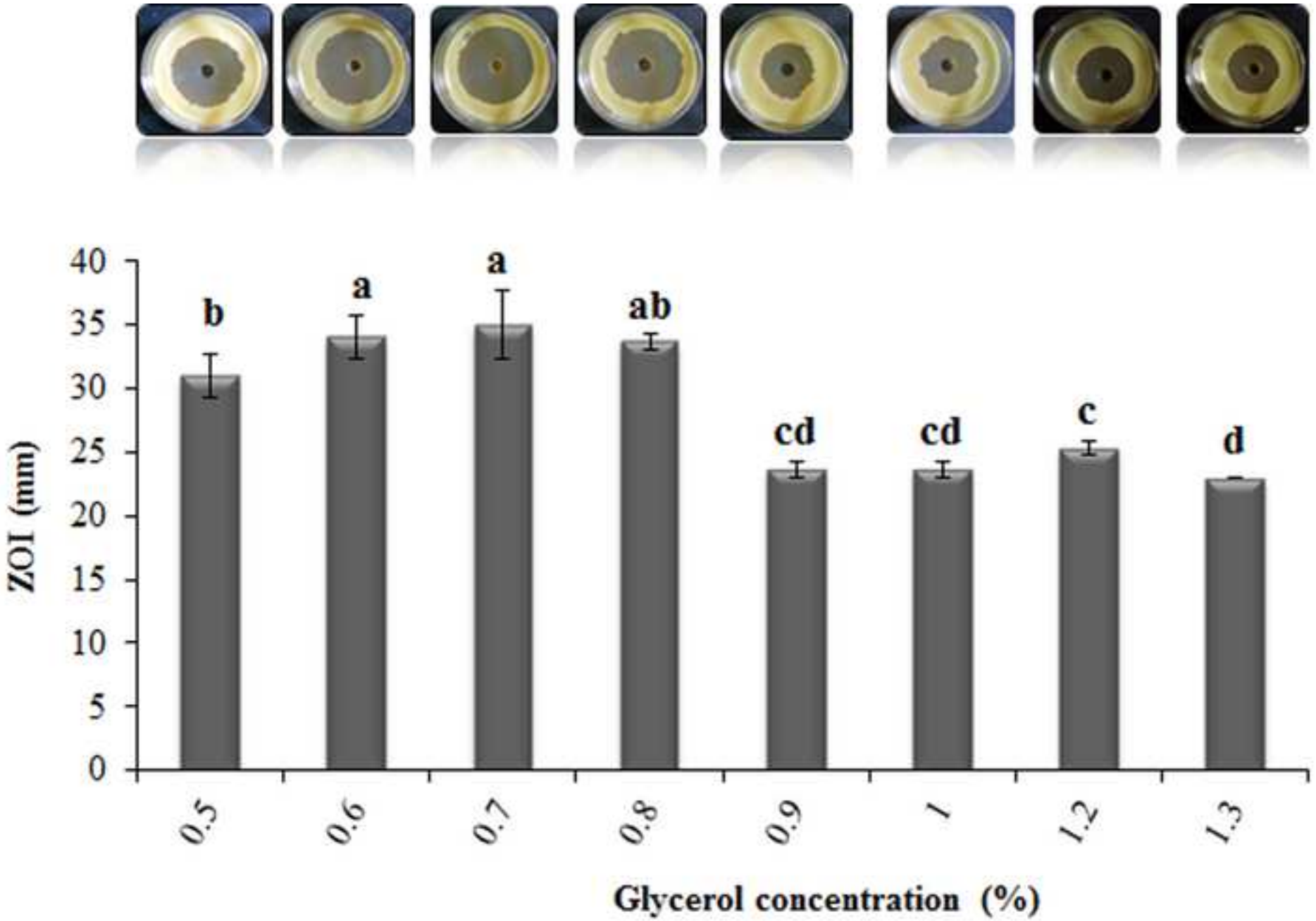

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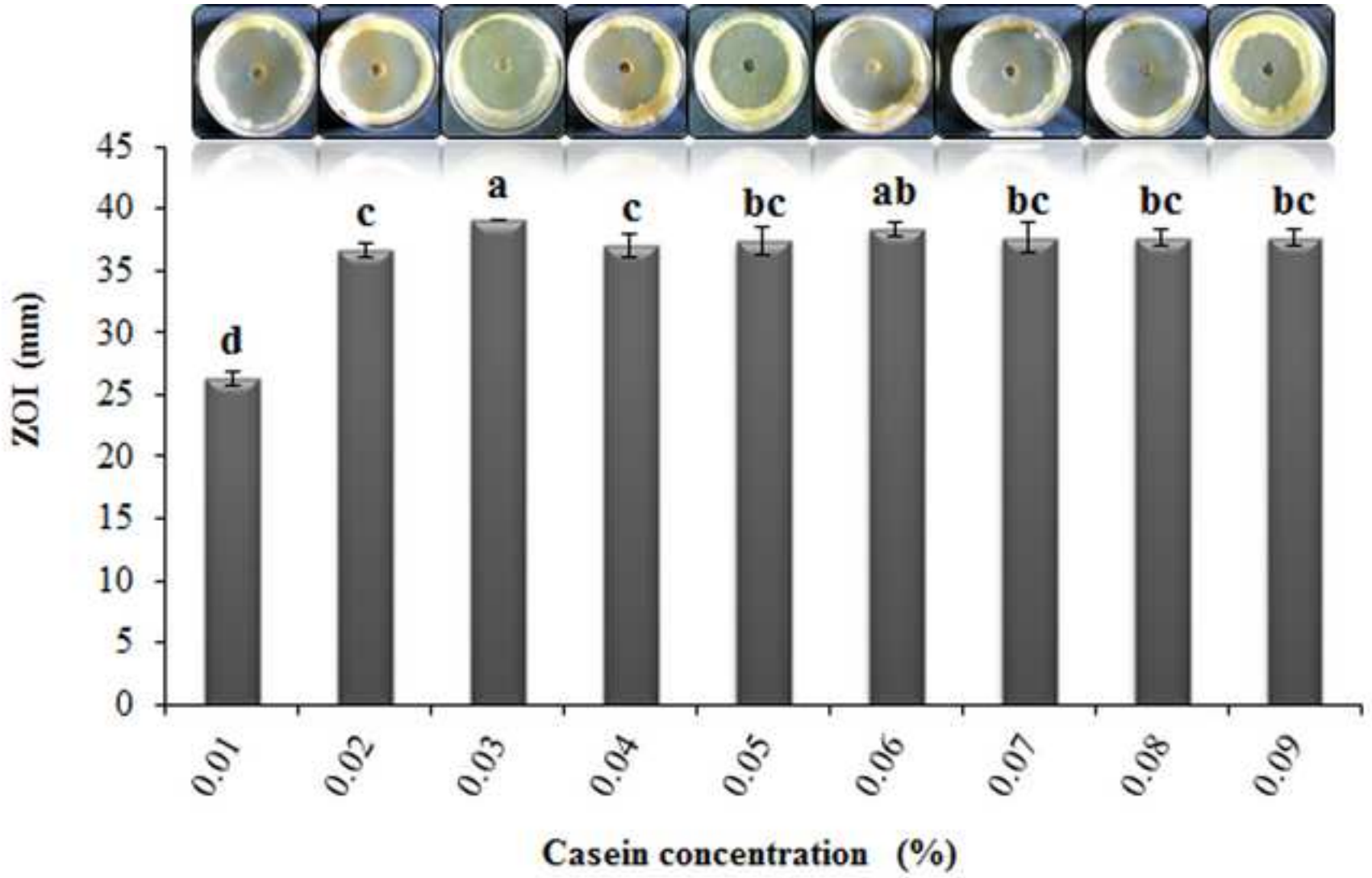

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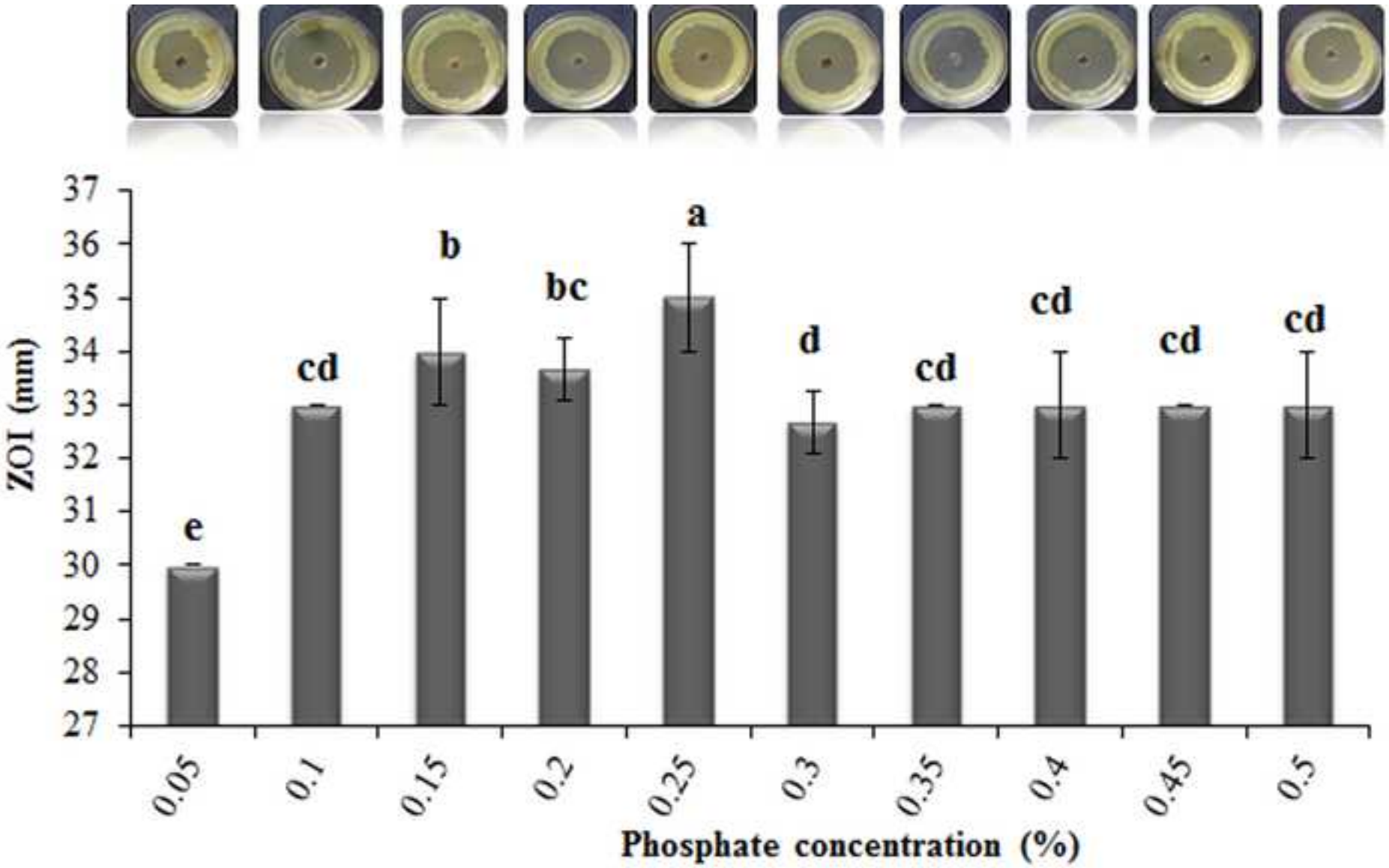

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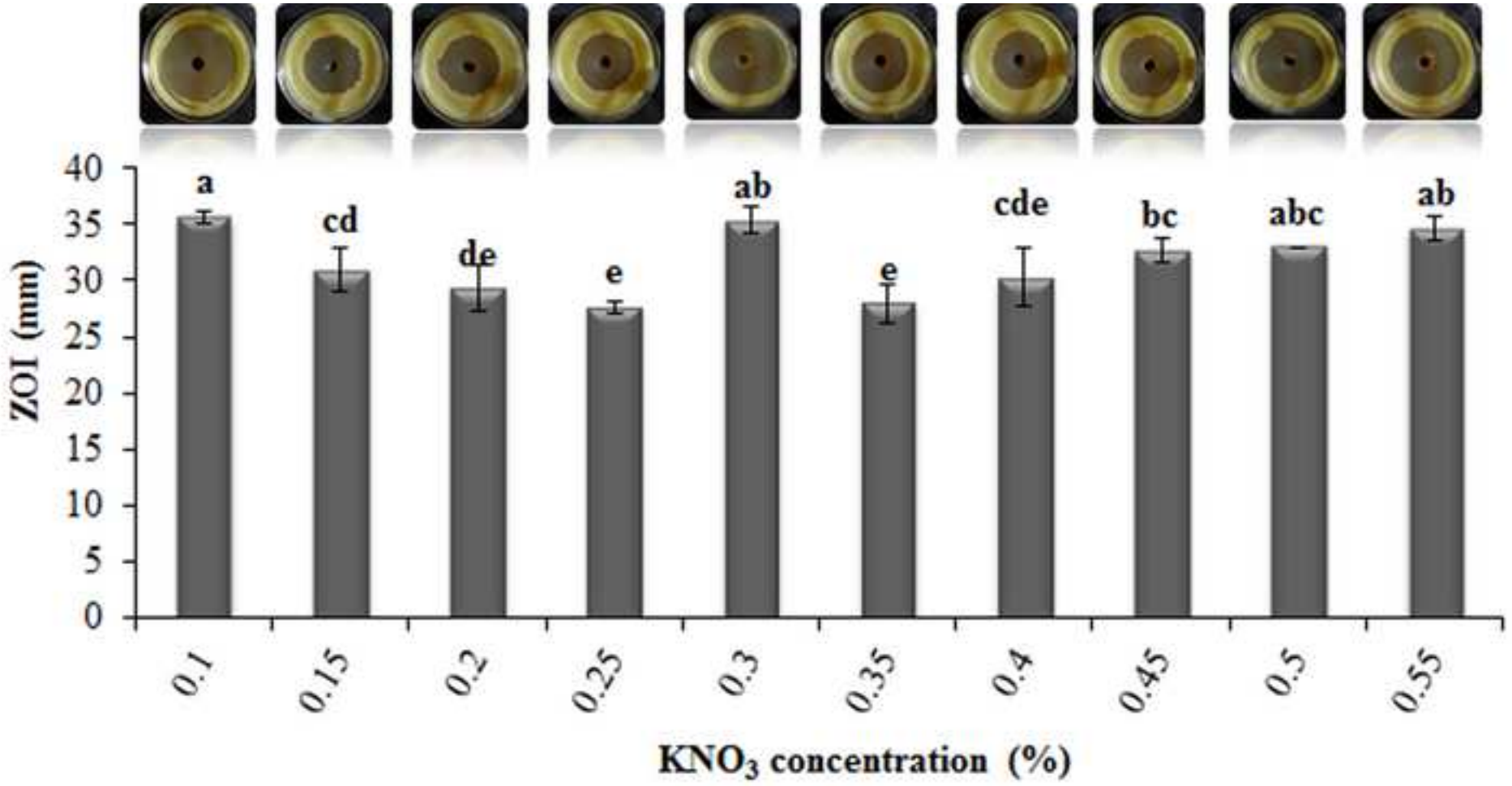

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