PROGRESS IN BIOLOGICAL SCIENCES

Effect of single additive bivalent metal ions on the growth of *Streptomyces clavuligerus* and clavulanic acid production

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Abstract

Metal ions are the main impurities of water and media ingredients used in fermentation processes. In this research, the effect of Ca^{+2}, Co^{+2}, Cu^{+2}, Fe^{+2}, Mg^{+2}, Mn^{+2} and Zn^{+2} as chloride and sulfate salts was studied on the clavulanic acid production and *Streptomyces clavuligerus* growth. All chloride salts had negative effect on clavulanic acid production and no clavulanic acid was produced in the media containing more than 1.5 mM $ZnCl_2$ and 18 mM $FeCl_2$. $CuCl_2$ and $CaCl_2$ increased biomass production, while the other chloride salts decreased it. Concentration of clavulanic acid in the 0.2 mM $MnSO_4$ containing medium was 1.21 times more than that of control. There was no significant difference in antibiotic concentration in the medium containing 0.41 mM $MgSO_4$ and control. Other sulfate salts decreased antibiotic production. $MgSO_4$, $CuSO_4$ and $FeSO_4$ increased the biomass, while other sulfate salts decreased it. Minimum and maximum specific consumption rate of glycerol were seen in the medium containing $CuSO_4$, and $MnSO_4$, respectively.

Keywords: β-lactamase inhibitor, Clavulanic acid, Metal ions, *Streptomyces clavuligerus*, Fermentation water quality

Introduction

Clavulanic acid (CA) is a potent broad-spectrum β-lactamase inhibitor produced by *Streptomyces clavuligerus*. Commercial formulation of CA such as Augmentin™ and Timentin™ that are the combinations of CA together with amoxicillin or ticarcillin, respectively, have made CA a product valued in excess of a billion dollars/annum (1) and created a powerful incentive to increase the yield of the process by various strategies including, genetic manipulation of the strain (2, 3), optimization of the media formulation (4), fermentation process condition (5) and down-stream processes (6).

Complex media, which contain agricultural products such as soy meal (1) and peanut meal (4) give a higher antibiotic yield than chemically defined media and are used for the production of antibiotics in industry. However, these complex substrates and the water used to make fermentation media, contain unknown concentrations of metal ions.

Metal ions act as co-enzymes in clavulanic acid biosynthesis. Carboxyethyl arginine β-lactam synthetase, proclavaminate amidinohydrolase and clavaminate synthase, are three metallo-enzymes of the clavulanic acid biosynthetic pathway which require Mg^{2+} (7), Mn^{2+} (8) and Fe^{2+} (9), respectively. To avoid batch-to-batch variability of yields, it is necessary to define concentration limits for each ion. Catalytic effect of different ions on the hydrolysis of CA in aqueous solution was reported, previously (10), however, according of our knowledge, no research work has been done on the sensitivity of *S. clavuligerus* to different concentrations of metal ions.

The goal of this research is to study the effect of some metal ions on the growth of *Streptomyces*

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clavuligerus in a complex industrial medium and to find suitable metals, their suitable forms and ranges for clavulanic acid production.

Materials and methods

Bacterial strain and media

Streptomyces clavuligerus DSM 738 (UTMC0021) was the strain used throughout the study. The sporulation medium used was ISP2agar, containing (per liter): glucose 4 g, malt 10 g, yeast extract 4 g, $CaCO_3$ 2 g, agar 12 g. The composition of the seed medium used was (per liter): peptone 10 g, malt powder 10 g, glycerol 20 g, deionized water 1000 ml, pH 7.0± 0.1 (11). The composition of the fermentation medium used was (per liter): dextrin 1 g, soluble starch 20 g, glycerol 3 g, soy flour 10 g, deionized water 1000 ml, pH 7.0± 0.1.
Elemental analysis of the fermentation medium was done by quality control laboratory, Shifa-Farmed, Biotechnological Industrial Group, Tehran, Iran.

Salts added to the medium

Various concentrations of $CaCl_2$, $MnCl_2.4H_2O$, $FeCl_2.4H_2O$, $MgCl_2.6H_2O$, $CuCl_2.2H_2O$, $CoCl_2.6H_2O$, $ZnCl_2$, $CaSO_4.2H_2O$, $MnSO_4.H_2O$, $FeSO_4.7H_2O$, $MgSO_4.2H_2O$, $CuSO_4.5H_2O$, $CoSO_4.7H_2O$ and $ZnSO_4.7H_2O$ were used. All salts were purchased from Merck. Salt solutions were dissolved in deionized water, sterilized by 0.22µm bacteriological filters and added to the autoclaved fermentation media to avoid the interaction of metal ions with the media components.

Cultural method

The amount of 1 ml of spore suspension (*ca*. 10^7-10^8 spores per ml) of *S. clavuligerus* DSM 738 was inoculated in 500 ml Erlenmeyer flasks containing 100 ml of seed medium. The flasks were incubated at 28°C on a rotary shaker at 220 rpm for 18-20 h. one ml of the seeding medium was added to each fermentation flask (100 ml Erlenmeyer flasks, each containing 20 ml medium). The flasks were incubated in a rotary shaker at 220 rpm for 3 days at 28°C.
All experiments were performed in triplicates in three batches and the results were reported after one-way analysis of variance and Tukey HSD test.

Assays

The samples were kept at -70ºC for further analysis, after measuring the pH.

Biomass

Dry cellular weight (DCW) was measured by heating 5 ml of the fermentation samples in for 24 hours at 75°C.

Residual glycerol

Glycerol was measured by the colorimetric method (12). This method is based on the periodate oxidation of glycerol and the formation of formaldehyde and the absorbance was measured at 412 nm.

Residual sugures

The total sugars were assayed by the phenol-sulfuric method (13).

Clavulanic acid

Concentration of clavulanic acid in the fermentation broth was determined by HPLC (14). This method is based on measuring the absorbance of immidazole derived products of clavulanic acid at 311 nm. The HPLC system (Adept 4900; Cecil; UK) was equipped with a UV detector (CE4200; Cecil). AC18 (250×4.6 mm, Hichrom, UK) column was used; the mobile phase consisted of methanol (30%, flow rate: 0.147 ml/min) and 50 mM phosphate buffer (70%, flow rate: 0.348 ml/min). Column temperature, 27ºC; sample injection volume, 20 µl. Standard potassium clavulanate was provided by Kosar pharmaceutical Co., Tehran, Iran.

Results

Effects of ions on the clavulanic acid production

Chloride salts

The effect of metals as chloride salts on the clavulanic acid production was shown in Figure 1. All metal chloride salts had negative effect on clavulanic acid production by *S. clavuligerus* DSM 738 (P<0.05). Even in their least concentrations studied, concentration of clavulanic acid production in the media containing chloride salts was less than that of the control (without any metal salt). No clavulanic acid was produced in the media

containing 1.5-15 mM $ZnCl_2$ and 18-90 mM $FeCl_2$. On the other hand, addition of $CuCl_2$ and $CaCl_2$ to the fermentation media increased the biomass concentration, while the other chloride salts decreased the growth of *S. clavuligerus* DSM 738 (P<0.05).

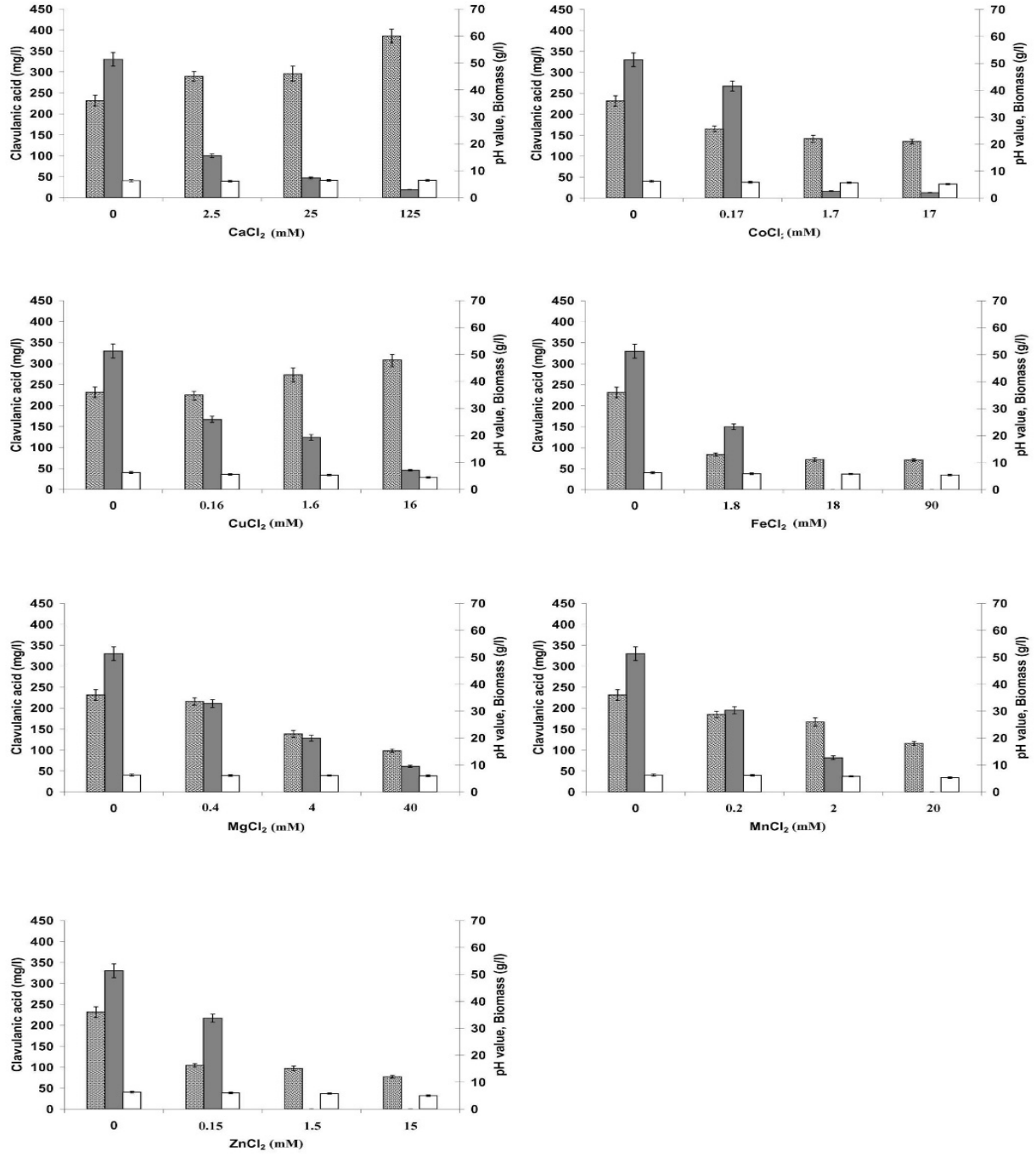

Figure 1. The effect of various metals as chloride salts on clavulanic acid production by *Streptomyces clavuligerus* DSM 738 in the basal medium containing various concentrations of the metal salts. A control fermentation medium in the absence of any metal supplement was also included (▩biomass (g/l), ■clavulanic acid, □ pH).

Sulfate salts

The effect of metals as sulfate salts on the clavulanic acid production was shown in Figure 2. Only $MnSO_4$ increased clavulanic acid production. The difference of antibiotic concentration in the media containing 0.41 mM Mg^{2+} and control was not significant ($P<0.05$). In the media containing Ca^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}, less clavulanic acid was produced than that of control. Addition of $MgSO_4$, $CuSO_4$ and $FeSO_4$ increased the biomass concentration, while other metal sulfate salts decreased it, significantly. In the media containing sulfate and chloride salts, pH of the fermentation media was more and less than that of control, respectively.

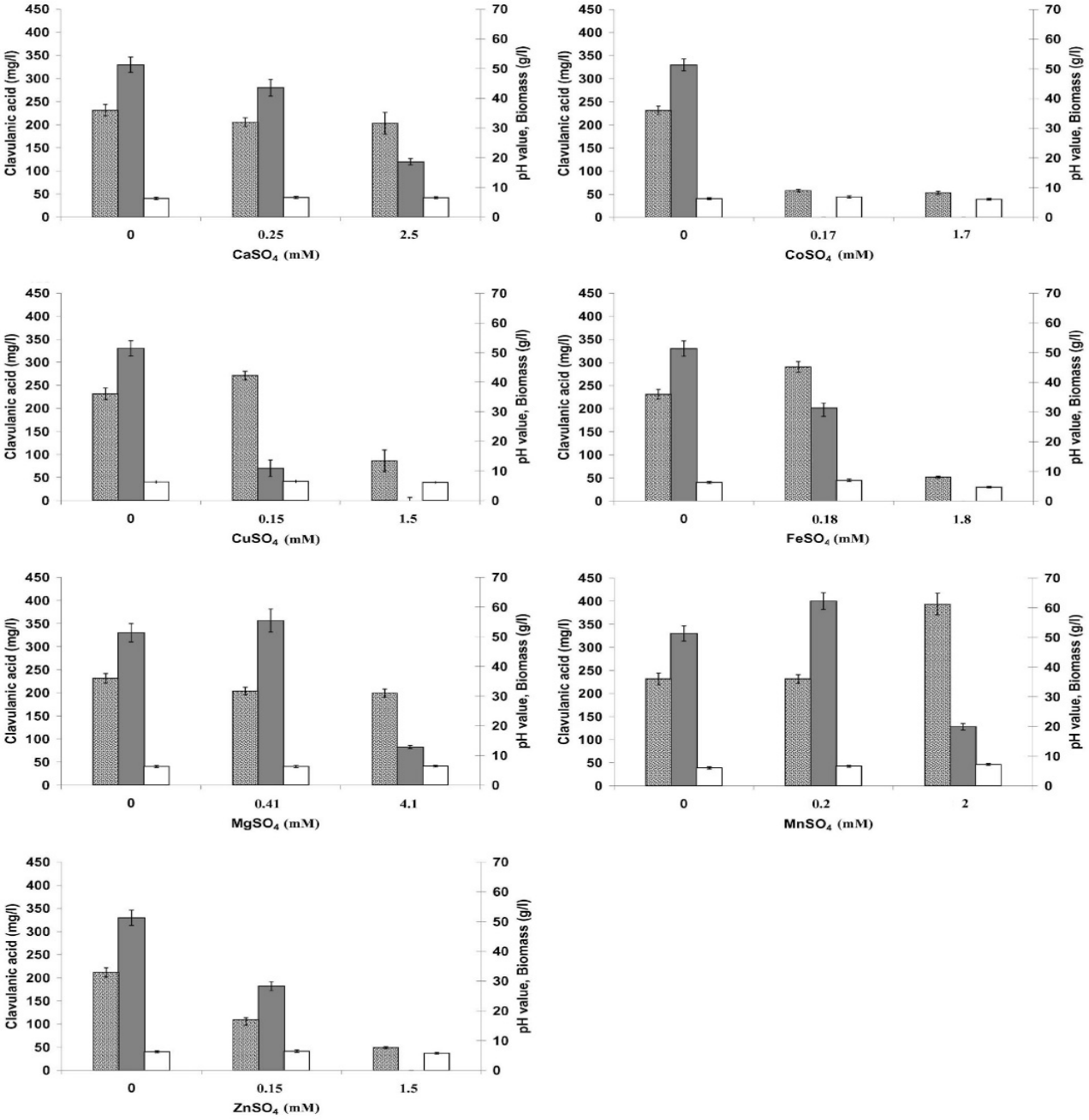

Figure 2. The effect of various metals as sulfate salts on the clavulanic acid production by *Streptomyces clavuligerus* DSM 738 in the basal medium containing various concentrations of the metal salts. A control fermentation medium in the absence of any metal supplement was also included (▩biomass (g/l),■clavulanic acid, □ pH).

Effect of ions on the morphology of *Streptomyces clavuligerus* DSM 738

Addition of metal salts affected the morphology and life span of the *Streptomyces clavuligerus* DSM 738. Two main morphologies were seen in the fermentation media (Fig. 3). In the control and the media containing all sulfate salts of the metals (except in the media containing $CoSO_4$ and 1.8 mM $FeSO_4$) and the media containing $CaCl_2$, $CuCl_2$ and 0.4 mM $MgCl_2$, the hyphae were long and without branching. But, in the media containing $CoCl_2$, $CoSO_4$, $FeCl_2$, 4 mM $MgCl_2$, 1.5 mM $ZnCl_2$, 1.8 mM $FeSO_4$, the hyphae were short. However, some irregular morphologies were seen. In the media containing 0.2-2 mM $MnCl_2$ and 0.15 mM $ZnCl_2$, the hyphae were long and with multiple branching (Fig. 3c). Although, in the medium containing 0.15 mM $ZnSO_4$, the hyphae were long and without branching, but some differentiated, long spiral hyphae were seen (Fig. 3d).

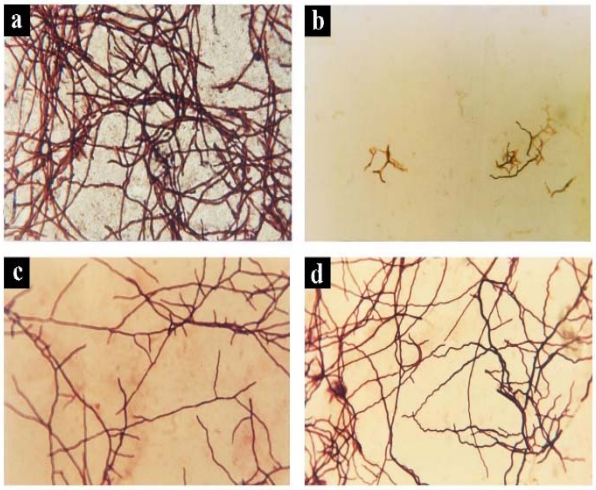

Figure 3. Effect of metals on the morphology of *Streptomyces clavuligerus* DSM 738, magnification ×1000. **(a)** Control medium, **(b)** medium containing 40 mM $MgCl_2$, **(c)** medium containing 0.2 mM $MnCl_2$ and **(d)** medium containing 0.15 mM $ZnSO_4$.

Effect of $MnSO_4$ and CuS_4 on the fermentation parameters of clavulanic acid production

As mentioned above, $MnSO_4$ and $CuSO_4$ increased the production of clavulanic acid and biomass, respectively. Therefore, these salts were chosen as a model for further study, and the results were shown in Figure 4. In the medium containing $CuSO_4$, less glycerol and more carbohydrate was consumed than that of other media. Minimum and maximum specific consumption rate of glycerol were observed in the $CuSO_4$ and $MnSO_4$ containing media, respectively. However, no significant correlation was seen between the addition of metal and specific consumption rate of total carbohydrates ($P<0.05$). At the end of fermentation, glycerol concentration in the medium containing $MnSO_4$ was zero, but 0.57% of glycerol was not consumed in the $CuSO_4$ containing medium. As shown in the table 1, specific growth rate (μ) of *Streptomyces clavuligerus* DSM 738 was the most in the $CuSO_4$ containing medium. Growth of the strain in the media containing $CuSO_4$ was faster than that of control and MnSO4 containing medium. In the first day, the hyphae of *Streptomyces clavuligerus* DSM 738 in the medium containing CuSO4, were longer and more branched than that of control and $MnSO_4$ containing medium, whereas they were at primitive stage of growth cycle in control and $MnSO_4$ containing medium. Profile of clavulanic acid production in the media containing metal salts and control was different. In the control, antibiotic acid concentration was decreased after the third day, but it increased in the $MnSO_4$ and $CuSO_4$ containing media. However, clavulanic acid gradient in the $MnSO_4$ containing medium was more than $CuSO_4$ containing medium.

Table 1. Effect of $MnSO_4$ and $CuSO_4$ on the fermentation parameters of clavulanic acid production, μ (specific growth rate), q_p (productivity), q_s (specific consumption rate for total carbohydrates), and $q_{s'}$ (specific consumption rate for glycerol).

Incubation time (h)	Medium	μ (h^{-1})	q_p (h^{-1})	q_S (h^{-1})	$q_{s'}$ (h^{-1})
4	Control	0.041	0.006	0.004	0.0003
	Mn^{2+}	0.042	0.006	0.001	0.0002
	Cu^{2+}	0.044	0.004	0.004	0.0001
48	Control	0.004	0.032	0.007	0.0009
	Mn^{2+}	0.002	0.032	0.006	0.0006
	Cu^{2+}	0.006	0.021	0.008	0.0001
72	Control	-0.011	0.192	0.003	0.0010
	Mn^{2+}	-0.009	0.190	0.002	0.0010
	Cu^{2+}	-0.008	0.060	0.004	0.0001
96	Control	-0.002	-0.206	0.002	0.0020
	Mn^{2+}	-0.003	0.257	0.002	0.0030
	Cu^{2+}	-0.003	0.130	0.001	0.0010

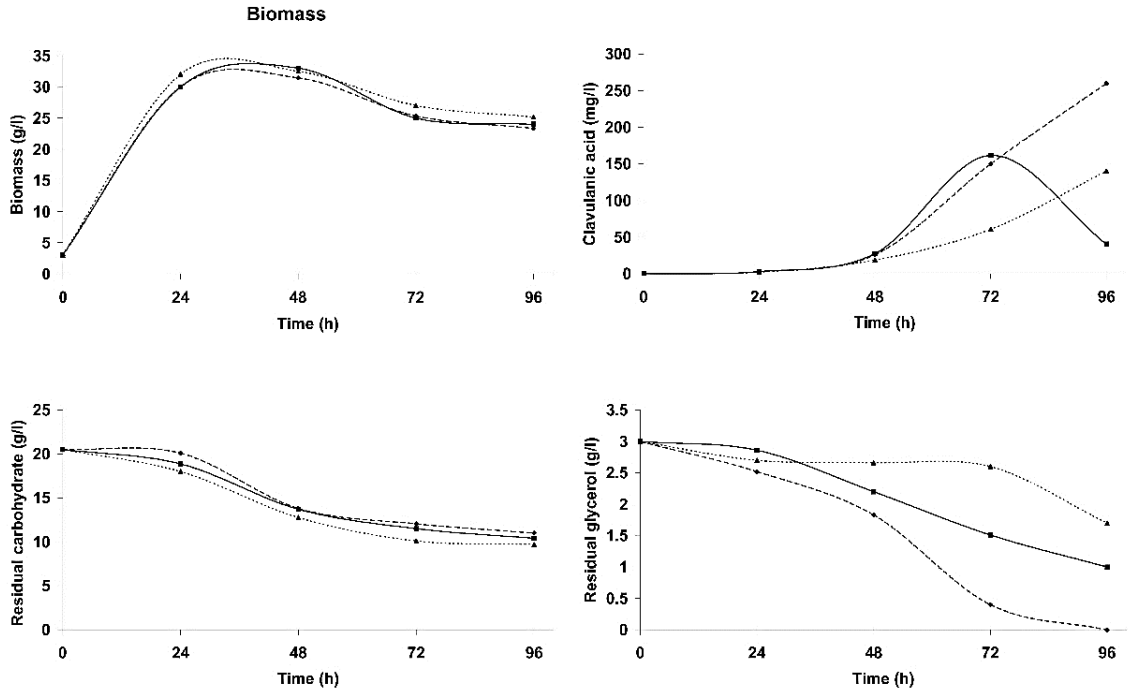

Figure 4. Effect of $CuSO_4$ and $MnSO_4$ on the growth of *S. clavuligerus* DSM 738, production of clavulanic acid production and substrate consumption. ▲, $CuSO_4$ containing medium; ♦, $MnSO_4$ containing medium and ■, control.

Discussion

Normally, sufficient quantities of metal ions, required by the fermenting microorganisms are present as impurities in the water supplies and in the other agricultural-based media ingredients (15). For example, soya flour and corn steep liquor contain a wide range of minerals that usually satisfies the minor and trace minerals needs. Occasionally, levels of metal ions, e.g. calcium and magnesium are to low to fulfill requirements and these may be added as specific salts. On the other hand, using of mineral rich water may contain extra concentration of metals, which may be toxic for the industrial strain. Elemental analysis of the basal fermentation medium is shown in Table 2.

The results obtained showed that sulfate was a suitable salt form for addition of a specific ion metal to the clavulanic acid production media, but addition of the metal as chloride was not recommended. Negative effect of the metal ions as chloride form, may be due to increasing of CA hydrolysis in the aqueous media containing chloride form of the metals (10). Therefore, level of chlorine in the water used for clavulanic acid fermentation has been considered. However, chloride form of salts was routinely used in the actinomycetes sporulation media (16).

The presented result was shown that addition of $CuSO_4$ increased the biomass concentration of *S. clavuligerus* DSM 738 and provoked hyphal branching. It was concluded that the addition of $CuSO_4$ to the seeding media can decrease the down-time of the process by reducing the time of seeding process. Enhancing effect of Fe^{3+} and Mg^{2+} on the growth of *Paenibacillus polymyxa* SQR-21, producer of a fusaricidin-type antifungal was reported (17).

Table 2. Elemental analysis of basal fermentation medium.

Minerals/Heavy Metals	mg/1000 ml
Ca	33.6
Mg	37.2
Mn	0.42
Na	1.2
Zn	0.55
Ni	1.02
Cd	0.006
Pb	0.005
Hg	0.003

As shown previously, addition of $MnSO_4$ to the fermentation media increased the concentration of

clavulanic acid; it may be due to an increase in clavulanic acid production by affecting the biosynthetic enzymes (8) or by decreasing of clavulanic acid degradation in the metal containing media. Decreasing effect of magnesium of the degradation of clavulanic acid has been reported (18). It can be concluded that addition of $MnSO_4$ in fed-batch regime can be use to enhance the yield of the antibiotic production. There is no report on the effect of metal ions on clavulanic acid production. However, effect of metal ions on the production of other antibiotic was reported, earlier. It was shown that Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{2+} had some promoting effect on the production of an antifungal antibiotic by *Streptomyces galbus* (19). Also, enhancing effect of ferrous ion on Iturin A, a lipopeptide antibiotic by *Bacillus amyloliquefaciens* B128 was reported (20). Positive effect of Fe^{3+} and Mg^{2+} on the production of a fusaricidin-type antifungal by *Paenibacillus polymyxa* SQR-21 was reported (17), too.

The presented results may be useful from biotechnological point of view. It can help to optimize the yield and quality of the biotechnological products, decrease the expenses of down-stream processing and to define the quality of water and media ingredients in the fermentation processes. However, on the base on the available date, we are not able to assign a specific role for the useful or toxic metals in the complex media. Further study, using chemically defined media by specific experimental design, in order to find out the role of metals and their interactions by other media ingredients is needed.

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