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Research Article

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Effect of Fe^{2+}, Mn^{2+} and Cu^{2+} ions on growth and soluble protein production in *Citrobatcer freundii* and *Klebsilla pneumoniae*

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ABSTRACT

Citrobacter freundii, is aerobic gram-negative bacilli. In the Biotech industry, C. freundii produces many important enzymes including phosphatase, α-galactosidase and selenocysteine beta-lyase. While Klebsiella pneumoniae is a nosocomial pathogen. In spite of its potential pathogenicity, it has several metabolic potentials, such as able to produce 1,3-propanediol dehydrogenase, 2,3-butanediol, guluronate layse and these could be used in biotechnology applications. The micro-nutrients act, as cofactors, required in micro quantity may lead excess production of desired proteins, enzymes and other value added products. The metal ions may also act on metabolic pathway of gene expression, protein folding and enzymatic activity in cells. In this study pH and temperature of C. freundii and K. pneumoniae were optimized and microelements viz., Zn^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+} and Ti^{2+} ions used to investigate the effect on growth and soluble proteins production by C. freundii and K. pneumoniae. We observed that Fe^{2+} ions helped dramatically in the growth of K. pneumoniae at concentrations of 60mg/ml and 80mg/ml (maximum) while Mn2+ at 60mg/ml. Therefore, Fe^{2+} and Mn^{2+} ions may be used in medium for promoting the growth and soluble protein production by K. pneumonia. The interesting outcome of this study on K. pneumoniae is that Ti^{2+} ions did not allowed increase in growth at none of the concentrations. Therefore, it may be used as anti-pneumoniae agent. 80mg/ml (maximum) followed by 60mg/ml of Fe^{2+} and Cu^{2+} ions in medium helped growth of C. freundii progressively. Hence, Fe^{2+} and Cu^{2+} ions may be used for promoting the growth. While Ti^{2+} and Zn^{2} ions could be used as antibacterial agent against C. freundii as they inhibited growth Therefore, 80mg/ml of $FeSO_4$ and $ZnSO_4$ may be used in medium to increase growth and soluble protein production.

Key words: *Citrobatcer freundii, Klebsilla pneumoniae*, microelements, metal ions, growth.

INTRODUCTION

Microorganisms are the most promising sources for large sale enzyme production. They can be easily grown and it is usually not difficult to scale up the production process. With microbes, it is possible to increase the production by changes in the growth conditions. These enzymes are used in industry as well as in medical applications. In the Biotech industry, *C. freundii* produces many important enzymes. The first enzyme produced by *C. freundii* is phosphatase apart from phosphatase it also produces 1,3-propanediol dehydrogenase [1] α-galactosidase and selenocysteine beta-lyase. The phosphatase activity of *C. freundii* has been also discovered to have resistance to some diagnostic reagents [2]. This activity has been postulated to be involved in lead accumulation, which can have

play an important role in the Biotech industry. It also produces class 1 AmpC cephalosporinase which can hydrolzye inactivate new cephamycins and cephalosporins [3].

K. pneumoniae is a widely recognized genus of opportunistic pathogenic bacteria. *K. pneumoniae* is the most important species of the genus in medical terms, and it is also very ubiquitous in nature, being present in surface water, soil, plants, and also as a saprophyte over the mucuses and intestine of mammals [4]. In spite of its pathogenic properties, *K. pneumoniae* has a complex metabolism that may lead to potential biotechnological applications such as production of 1,3-propanediol dehydrogenase [5] 2,3-butanediol, and guluronate layse [6]. 1,3-Propanediol (1,3-PD) is a bifunctional organic compound can also be used in the production of cosmetics, foods, lubricants, and drugs. The cellular response in the presence of metals includes various processes such as biosorption by cell biomass; active cell transport, binding by cytosolic molecules, entrapment into cellular capsules, precipitation and oxidation-reduction reactions [7] as well as protein-DNA adduct formation and induction of stress proteins [8].

The metal ions i.e. micro-nutrients usually act as cofactors and may lead growth, inhanced gene expressions, essential enzymatic reactions and metabolic pathways in the cell. These cofactors play essential and critical role in regulation of protein formation and other value added products. The usual cations that qualify as essential trace elements in bacterial nutrition are Mn, Co, Zn, Cu, Mo, Fe, Mg, Ni, K, Se, V, B, and Na.

Zinc is involved in a wide variety of cellular processes. Consequently, the ability to maintain the intracellular Zn^{2+} ion concentration within very narrow limits is a fundamental property of all living cells. It is required for maintaining the structural stability of macromolecules and it serves as a cofactor for more than 300 enzymes [9]. Zn^{2+} ion also plays a prominent role in gene expression and as a structural component in a large number of Zn^{2+}-dependent transcription factors. However, in excess it can inhibit the aerobic respiratory chain, have significant toxicity and act as a potent disrupter of biological systems. In prokaryotes the major mechanisms that maintain cellular Zn^{2+} ion concentrations are limited to the highly regulated processes of Zn^{2+} ion import, metal ion sequestration by metallochaperones and Zn^{2+} ion export across the cytoplasmic membrane [10]. Cells respond to excess Zn^{2+} by metal-inducible resistance mechanisms. Zn^{2+} resistance in bacteria is mainly based on active efflux of metal ions to prevent toxic effects in the cell. The efflux of Zn^{2+} is facilitated by P-type ATPases, CBA transporters and CDF chemiosmotic transporters.

In the presence of copper, the significant induction of citric acid overflow was observed, while concomitantly lower levels of total lipids were detected in cells. Its effect was more obvious in a medium with Mg^{2+} as sole divalent metal ions while addition of Cu^{2+} have less pronounced effect. When 0.1 mM concentration Fe^{2+} and Zn^{2+} is added in the medium inhibition in malic enzyme activity was observed in fungal cells but 0.1mM concentration of Cu^{2+} ions completely eradicate malic enzyme activity. Fungus can cells tolerate higher concentrations of Zn^{2+} in the presence of NaCl at 37°C than at 25°C [11].

The problems which are being faced by Biotechnology Company are the unavailability of data regarding different micro-elements and their proper concentration for the optimal growth and production. In the current study, the microelements which have important role in the growth promotion and the production of proteins have been evaluated.

EXPERIMENTAL SECTION

The strains of *K. pneumoniae* and *C. freundii* were collected from Department of Biotechnology, Jiwaji University, Gwalior (M.P.). The strains were streaked on Muller Hinton's Agar (Himedia Pvt. Ltd.) Medium plates and incubated for 24hrs at 37ºC.

Determination of optimum growth conditions: For optimum growth of the bacterial cultures, two parameters *i.e.*, temperature and pH were considered. 25ml Muller Hinton's Broth (Himedia Pvt. Ltd.) was added in 4 sets, autoclaved and inoculated with 20µl of freshly prepared culture of each bacterial strain by overnight growth at 37ºC in MH broth. The four sets of flasks were incubated at 25ºC, 30ºC, 35ºC, 37ºC and 40ºC. After an incubation period of 16hrs, their absorbance was taken at 595nm using a UV-1700 UV/Vis Spectrophotometer (Shimadzu, Japan). For determination of optimum pH, test tubes having 5ml MH broth were prepared in 5 sets and their pH were adjusted at

5.0, 6.0, 7.0, 8.0, and 9.0. These tubes were inoculated with 20µl freshly prepared culture of both bacterial strains. After an incubation period of 16h, their absorbance was taken at 595nm.

Effect of metal ions cofactors on bacterial growth: Metal cofactors viz., Zn^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+} and Ti^{2+} were used to evaluate their effect on the growth and total soluble protein production in *C. freundii* and *K. pneumoniae*. 20mg, 40mg, 60mg, 80mg and 100mg per litre were added in 50ml MH broth. 50µl of freshly prepared culture of both strains were added and incubated for 24hrs at 30ºC and 37ºC for *K. pneumoniae* and *C. freundii*, respectively in an incubator shaker at 125 rpm. After 24hrs incubation the OD was taken at 595nm.

Estimation of Protein: The concentration of total soluble protein secreted in the MH medium by the strain of *C. freundii* was determined by Folin-Lowry method at 660nm using U UV-1700 UV/Vis Spectrophotometer (Shimadzu, Japan). BSA standard solution was used to estimate the soluble protein concentration with different treatments.

RESULTS AND DISCUSSION

Optimum pH and Temperature: The most optimum temperature for the growth of *K. pneumoniae* was found to be 30°C while the suitable temperature for growth of *C. freundii* was found 37°C. *K. pneumonia* showed optimum growth at pH=7 while *C. freundii* showed optimum growth at pH=5 (Figure 1 and 2). Under these optimum conditions of temperature and pH the growth curve also produced.

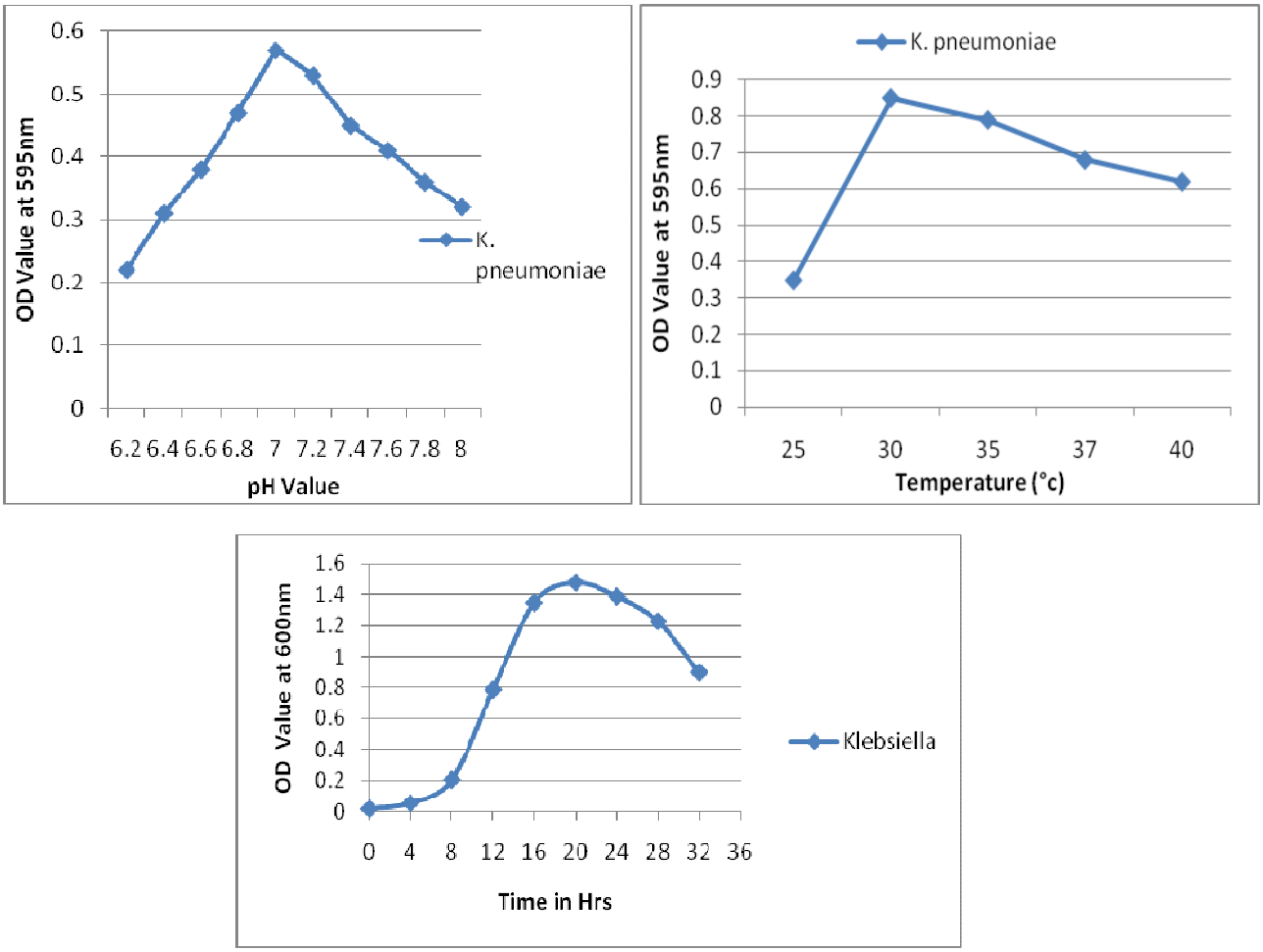

Figure 1: (a) Effect of pH on gowth of *K. pneumoniae*, (b) Effect of temperature on gowth of *K. pneumoniae*, (c) Growth curve of *K. pneumoniae* at pH=7.0 and temperature = 30ºC

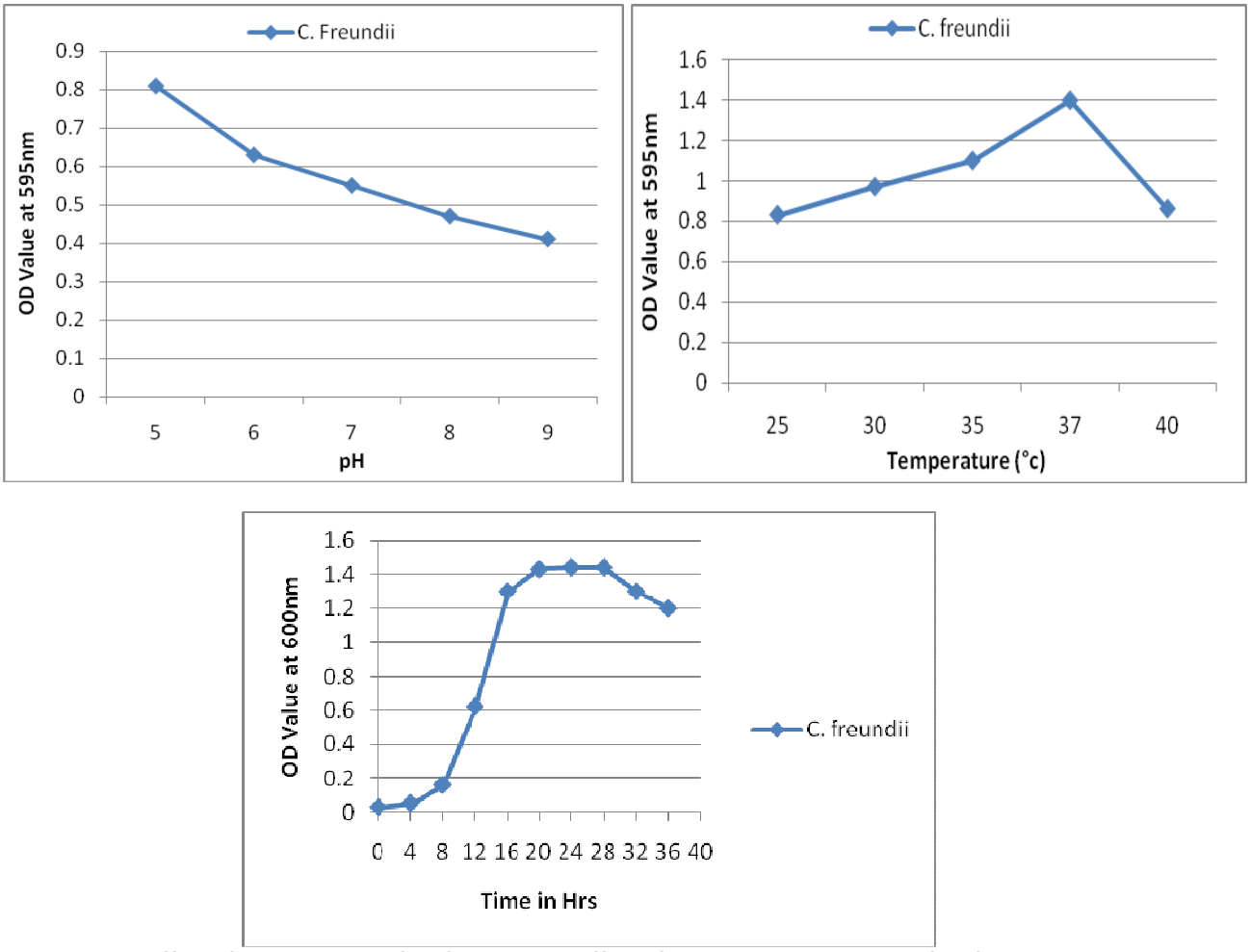

Figure 2: (a) Effect of pH on gowth of *C. freundii*, (b) Effect of temperature on gowth of *C. freundii*, (c) Gowth curve of *C. freundii* at pH=5 and temperature =37°C.

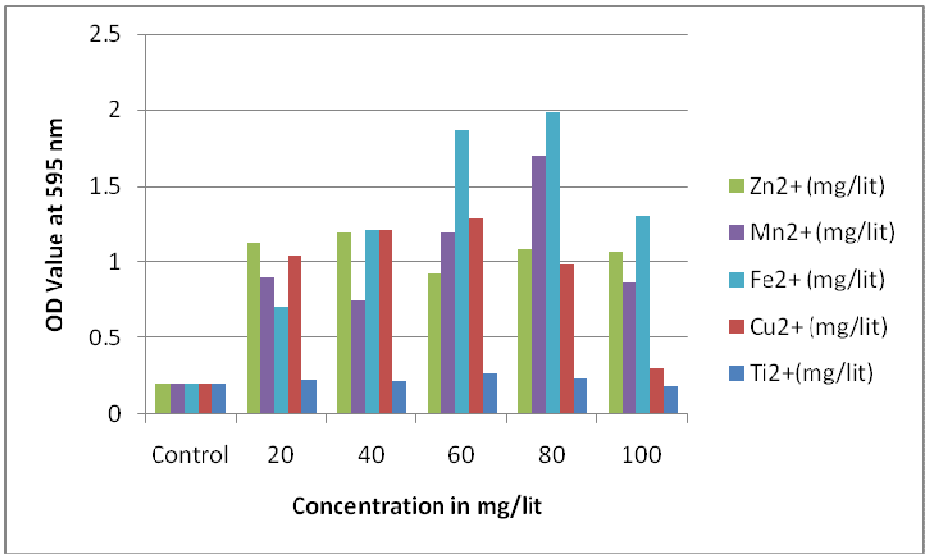

Figure 3. Comperative growth of *K. pneumoniae* in different metal ions

***Effect of microelements on K. pneumoniae*:** The Fe^{2+} metal ions helped dramatically in the growth of *K. pneumoniae* at concentrations of 60mg/lit and 80mg/lit (maximum). While at low concentrations ≤40mg/ml does affect growth intermediately. Mn^{2+} is the second metal ion that helped in the growth at 80mg/lit while at low concentration i.e. ≤ 60mg/lit it helped significantly. But the higher concentration i.e. 100mg/lit inhibited the growth. The Cu^{2+} and Zn^{2+} ions also helped intermediately. The interesting outcome of this study is that Ti^{2+} ions does not allowed the growth of *K. pneumoniae* at none of the concentration as compared with other metal ions and standard too. Therefore, it could be used as antibacterial agent against *K. pneumoniae.* While Fe^{2+} and Mn^{2+} may be used for promoting the growth of K. *pneumoniae* (Figure 3).

***Effect of microelements on C. freundii*:** The Fe^{2+} and Cu^{2+} metal ions helped dramatically in the growth of *C. freundii* at concentrations of 80mg/lit (maximum) followed by 60mg/lit. While at low concentrations ≤40mg/ml does affect growth significantly. Mn^{2+} metal ions affected growth at 60mg/lit but no significant growth was observed at other concentrations. Ti^{2+} and Zn^{2+} metal ions does not helped in the growth but they inhibited growth of *C. freundii* significantly. The interesting outcome of this study is that Ti^{2+} and Zn^{2} ions could be used as antibacterial agent against *C. freundii* while Fe^{2+} and Cu^{2+} may be used for promoting the growth (Figure 4).

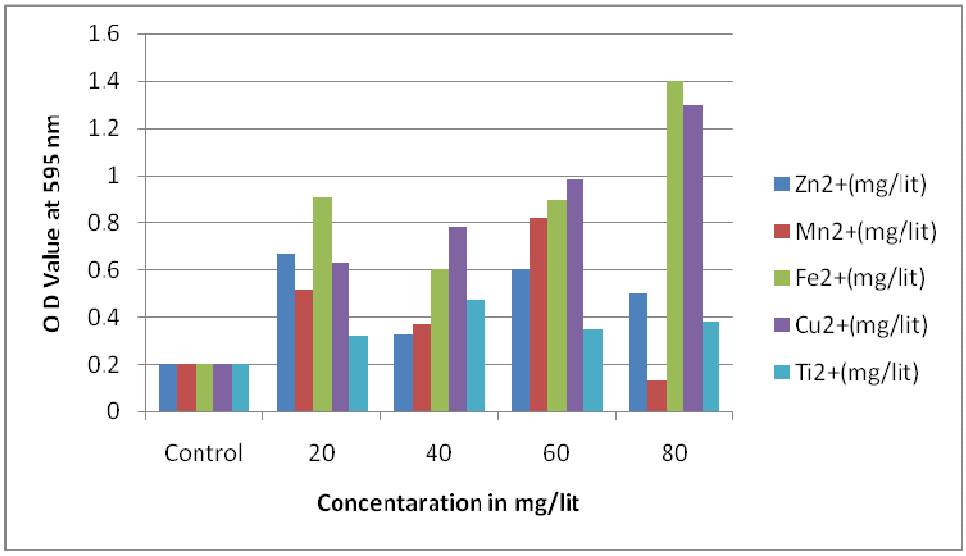

Figure 4. Comperative growth of *C. freundii* in different metal ions

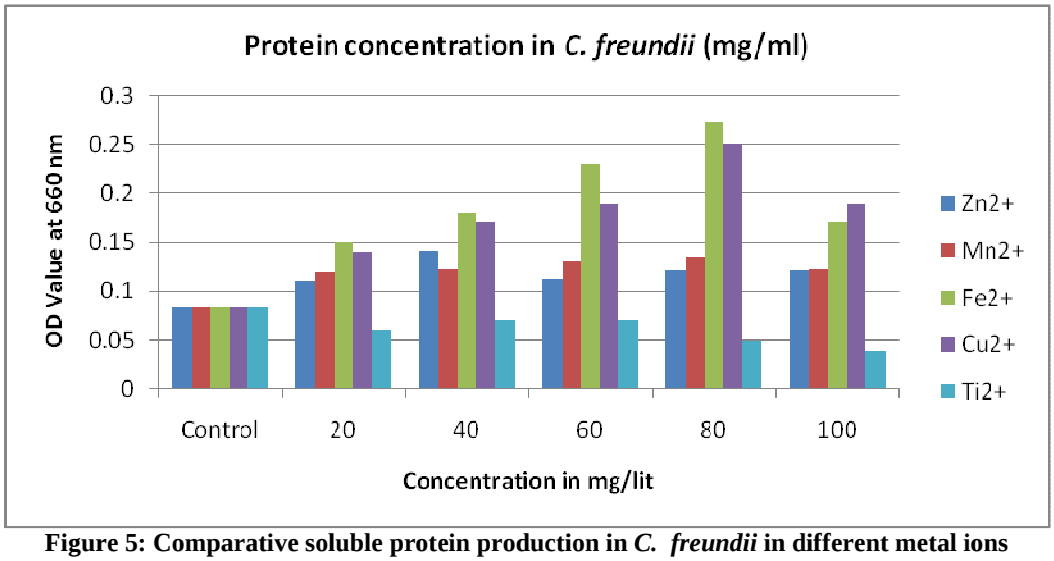

Figure 5: Comparative soluble protein production in *C. freundii* in different metal ions

Soluble protein production by *C. freundii*: Total soluble protein production was increased in all concentration of $FeSO_4$ and $CuSO_4$ progressively. The maximum protein production was observed in 80mg/lit followed by 60mg/lit of $FeSO_4$ and $CuSO_4$. $ZnSO_4$ and $MnSO_4$ showed significant increase in total soluble protein production but less than $FeSO_4$ and $CuSO_4$ (Figure 5). The concentration of $TiSO_4$ did not show any significant increase in total soluble protein production but it inhibited the production. It showed antibacterial activity. Therefore, 80mg/lit of $FeSO_4$ and $ZnSO_4$ may be recommended; as these are capable to increase growth and ultimately concentration of total soluble protein.

The total protein production increases in a regular manner is reflected in growth with a60mg/lit and 80mg/lit concentration of $FeSO_4$ and $CuSO_4$ microelements. So any cofactor which promotes total protein production but they doesn't promote growth in the same manner. Further research based on this project work may help the industries to understand the most important micro-elements and their suitable concentration for culture of *C. freundii* and *K. pneumoniae* and the production of proteins and value added products. This would lead the companies to design efficient strategy for maximum production of important enzymes and therapeutic proteins at industrial scale.

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