Studies to explore the impact of Carbon & Nitrogen source for Lincomycin production at shake flask level using Streptomyces lincolnensis

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***Abstract* - Lincomycin and its derivatives are antibiotics exhibiting biological activity against Gram-positive bacteria. The semisynthetic chlorinated lincomycin is used in the clinical practices. The importance of carbon and nitrogen sources and the ideal carbon and nitrogen sources were optimized. Different phosphates were studied and their concentration was optimized. The concentration of dextrose, soya flour and sodium nitrate was determined using full factorial design. ANOVA analysis showed that dextrose and soya flour were significant components which had a greater impact on yield. The overall yield of lincomycin was increased from 1g/l to 3.5 g/l.**

I. INTRODUCTION

Lincomycin belongs to the lincosamide group and was first isolated from soil actinomycete near Lincoln, Nebraska. (Stratton, 1998). Lincomycin and its derivatives form a medically important group of chemotherapeutics. The actinomycete is a streptomyces species called *Streptomyces lincolnensis.* Lincomycin possess good in vivo and in vitro potency against a number of gram positive microbes (Lewis *et al*. 1963). It has gained clinical acceptance as a major antibiotic for the treatment of diseases caused by Gram positive microbes. The lincosamide member contains 6,8-dideoxy-6-aminooctose lincosamine as the most characteristic constituent (Figure 1).

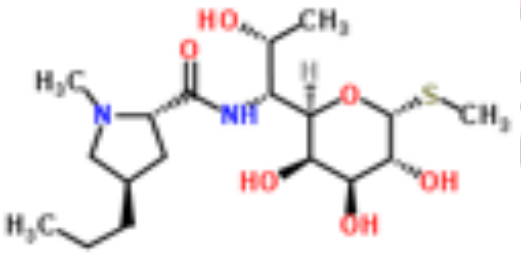

Figure 1: Structure of Lincomycin

The biosynthesis of most antibiotics is strongly regulated by the nature and concentration of carbon, nitrogen, phosphorus and the trace elements.

Production of lincomycin is carried out by secondary metabolite production through fermentation. Though fermentation is a very old process, production of the required quantity of product which meets the market needs is posing a big challenge((Dubey *et al*., 2008, 2011; Singh *et al*., 2009; Rajeswari *et al*., 2014).Media optimization is one of the most critically investigated phenomenon carried out during metabolite production. For designing a production medium, the most suitable fermentation conditions (e.g., pH, temperature, RPM, age.) and the appropriate medium components (e.g., carbon, nitrogen, salts etc.) must be identified and optimized accordingly. Thus by optimization of the media components we can achieve the desired concentration of the product.(Gupte and Kulkarni, 2003; Franco-Lara *et al*., 2006; Wang *et al.*, 2011). The flow sheet of media optimization in fermentation process is illustrated in figure 2 in a step by step manner.

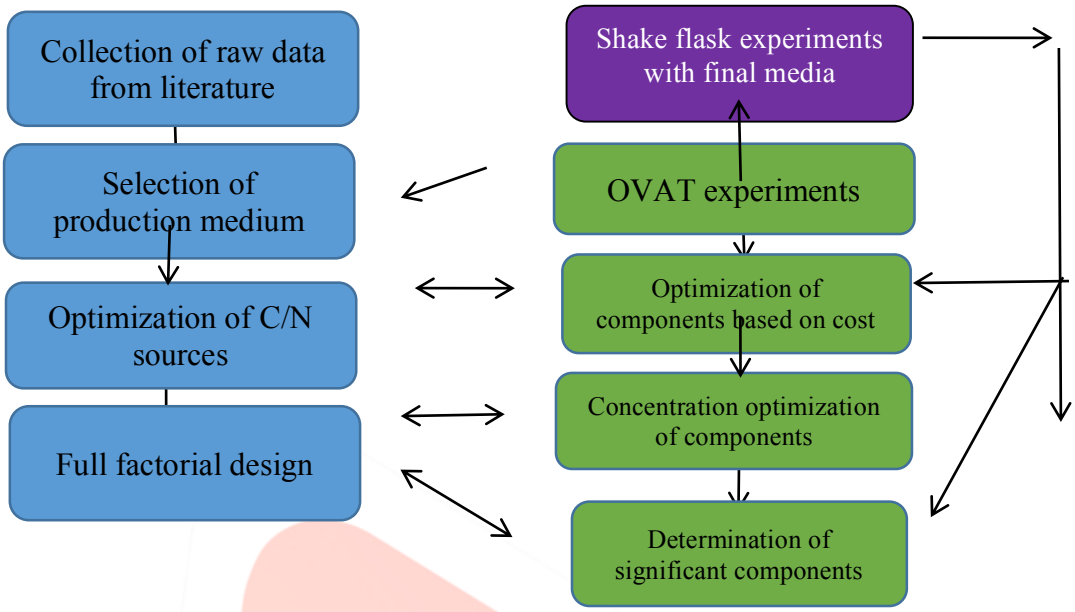

Figure 2: Media optimization flow sheet

In the current study the production medium for the Lincomycin production was optimized. The impact of various carbon and nitrogen sources on yield were studied. Different nitrates and phosphates were incorporated in the medium and checked for the desired yield The concentration of the optimized carbon and nitrogen sources was also optimized using full factorial design

Material and Methods

Microorganism

Streptomyces linconensis was used for the production of lincomycin in submerged fermentation. It is an actinomycete belonging to the genus *Streptomyces* The culture was maintained on slants and in the form of cryovials. The slant media comprised of soluble starch 19g/l, soya flour 5 g/l, Potassium nitrate 1g//l, ferrous sulphate 0.01 g/l, dipotassium hydrogen phosphate 0.5 g/l, magnesium sulphate 0.5 g/l, sodium chloride 0.5 g/l and agar 22 g/l. The slants after inoculation were incubated at 30°C for 6-8 days.

Growth media

Vertically cut half slant was inoculated in seed media containing 30 ml media in 250 ml erlenmeyer flask (10 g/l dextrose, 20 g/l CSL, 10g/l soluble starch, 2 g/l calcium carbonate pH 7.00). The flasks were incubated at 30°C at 240 rpm for 44-48 hrs. 10% of the grown seed was transferred to production containing 30 ml media in 250 ml erlenmeyer flask (soluble starch 40 g/l, dextrose 100 g/l, soya flour 10 g/l, ammonium sulphate 4 g/l, sodium chloride4g/l, diammonium hydrogen phosphate 0.2 g/l, sodium nitrate 8 g/l and calcium carbonate 8 g/l pH 6.2).The flasks were incubated at 30°C at 240 rpm.

Quantification by HPLC

Lincomycin produced in the culture broth was determined by HPLC. A mobile phase of methanol:water as 90:10 was used. 2.5g sample was weighed and dissolved in methanol, filtered and then injected. The resulting extracted solution was injected into the HPLC (Waters 2496) having C-18 column (Hypersil ODS, 5u C18 (250 mm X 4.6 mm) for the estimation of lincomycin. Concentration of lincomycin was calculated by comparison of peak areas with those standard lincomycin and subsequently lincomycin activity was calculated.

The carbon and nitrogen sources were optimized using one variable at a time method.

The suitable carbon and nitrogen source was then added to the medium to optimize the phosphates. Further 3 variable full factorial design was used to optimize the concentration of carbon and nitrogen sources.

Results and Discussion

Optimization of Carbon source

Carbon is the most important medium component, as it is an energy source for the organisms. It aids in the growth of the microbes and also helps in the production of secondary metabolites. The amount of carbon source utilization influences the formation of the desired product (Singh *et al*, 2016).

In the current study, Dextrose, sucrose and fructose were used as carbon source in the concentration of 150 g/l in production media. Table 1 shows the the different carbon source used along with the yield.

S no.	Carbon Source	Yield g/l
1	Dextrose	2.503

S no.	Carbon Source	Yield g/l
2	Sucrose	2.01
3	Fructose	0.982

Table 1: Carbon sources and their respective yield

According to table 1, media containing dextrose shows maximum yield whereas fructose shows very less yield. Dextrose supports the growth and product formation more as compared to the other carbon sources

Dextrose is available in two forms, powdered and syrup.Both were used to check the productivity.Table 2 represent the form of dextrose and their yield.

S no.	Component	Yield g/l
1	Dextrose	2.689
2	Dextrose syrup	2.606

Table 2: Forms of dextrose and its yield

Dextrose syrup shows similar yield as does dextrose powder. Dextrose syrup is more cost effective as compared to dextrose powder. Hence it was used in place of dextrose powder.

Optimization of Nitrogen source

Nitrogen sources are utilized by the microorganisms in the anabolic synthesis of cellular substances such as amino acids, purines, DNA and RNA.The antibiotic production is highly influenced by the type and concentration of nitrogen source. Ammonia, nitrate and amino acids are rapidly utilized by the microbial cells and can have an inhibitory effect on product formation. Thus, to avoid the inhibitory effect proper selection of nitrogen sources especially the ones that are released slowly can be used (Aharonowitz, 1980). Thus the selection of nitrogen source plays an important role in the fermentation process.

In the present study, the nitrogen source, casein, soya flour, corn steep liquor and yeast extract were used in the concentration 25 g/l. Table 3 shows the yield with the respective nitrogen source.

S no.	Nitrogen Source	Yield g/l
1	Casein	1.875
2	Soya Flour	1.950
3	Corn steep liquor	1.985
4	Yeast Extract	0.942

Table 3: Nitrogen sources and their respective yields

According to Table 3 it is observed that corn steep liquor and soya flour are more conducive for both the growth and Lincomycin production. Soya flour and corn steep liquor were used in combination and it was observed that the combination gave a better yield of 2.85 g/l as compared to individual nitrogen sources. In further experiments, inorganic nitrogen sources were optimized. Ammonium nitrate, sodium nitrate and potassium nitrate were added individually in each media in the concentration of 6g/l Table 4 shows the yield along with the inorganic nitrogen source.

S no.	Inorganic Nitrogen	Yield g/l
1	Ammonium nitrate	2.02
2	Potassium nitrate	1.425
3	Sodium Nitrate	2.07

Table 4: Inorganic nitrogen sources and their yield

It was observed that ammonium nitrate and sodium nitrate give similar yield whereas potassium nitrate has a negative impact on the production of lincomycin. In the present study, sodium nitrate was optimized as the inorganic nitrogen source. Ammonium nitrate was not used as it is not easily available due to safety constraints. Combination with other nitrogen sources was also tried but thought the cell mass obtained was considerable, the yield was very low than desired.

Optimization of Phosphates

Phosphorus is involved in both the primary and secondary biosynthesis (Sanchez, 2002). The addition of phosphorus in the medium helps in increased biomass formation. In certain cases, phosphorus inhibits the biosynthetic pathways but Lincomycin is an exception (Sanchez, 2002). In the current study various phosphates were incorporated in the media to check their impact on yield. Diammonium hydrogen phosphate, Dipotassium dihydrogen phosphate and Potassium dihydrogen phosphate were added in the concentration of 0.5 g/l in the production media.

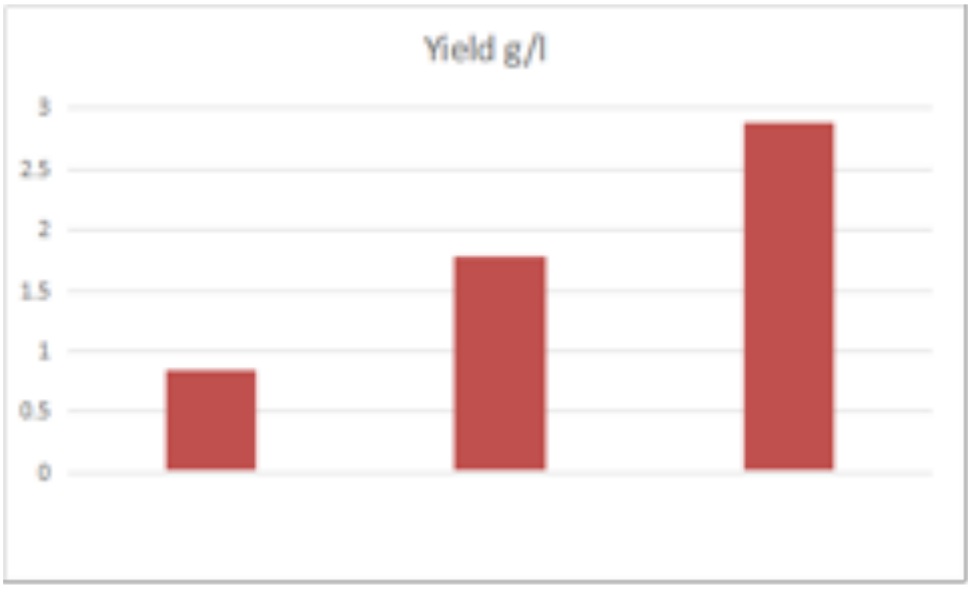

Figure 2: Phosphates and their respective yield.

According to figure 2, potassium dihydrogen phosphate gives maximum yield as compared the other phosphates used. Being a low pH buffer it helps in controlling the pH of the medium, during the process and thus enabling the biomass production and in turn product formation Further the concentration of potassium dihydrogen phosphate was increase from 0.5 g/l to 1.5 g/l. Figure 3 represents the effect of increased concentration of KH_2PO_4 on the yield and packed mycelial volume (growth).

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Figure 3: Concentration of KH_2PO_4 and the respective yield and PMV

Increasing the concentration does increase the growth represented here as packed mycelial volume whereas the yield decreases with increase in concentration. Thus the concentration of KH_2PO_4 was optimized to 0.5 g/l. Thus as phosphate is essential for growth, higher concentration causes pH imbalance and low Lincomycin yield.

Optimization of concentration of carbon and nitrogen source

Full factorial design is used to study the effect of each factor on the response variables, as well as the effects of interactions between factors of the response variable. A factorial design allows the effect of several factors and even interactions between them to be determined with the same number of trials as are necessary to determine any one of the effects by itself with the same degree of accuracy (Ronald fisher, 1926).Full factorial design with three variables, 8 run was performed to optimize the concentration of dextrose, soya flour and sodium nitrate.Table 5 shows the design of the full factorial along with the upper and lower limit.

Component	Lower Limit (g/l)	Upper Limit (g/l)
Dextrose	150	250
Soya Flour	15	35
Sodium nitrate	4	12

Table 5: Design of full factorial design

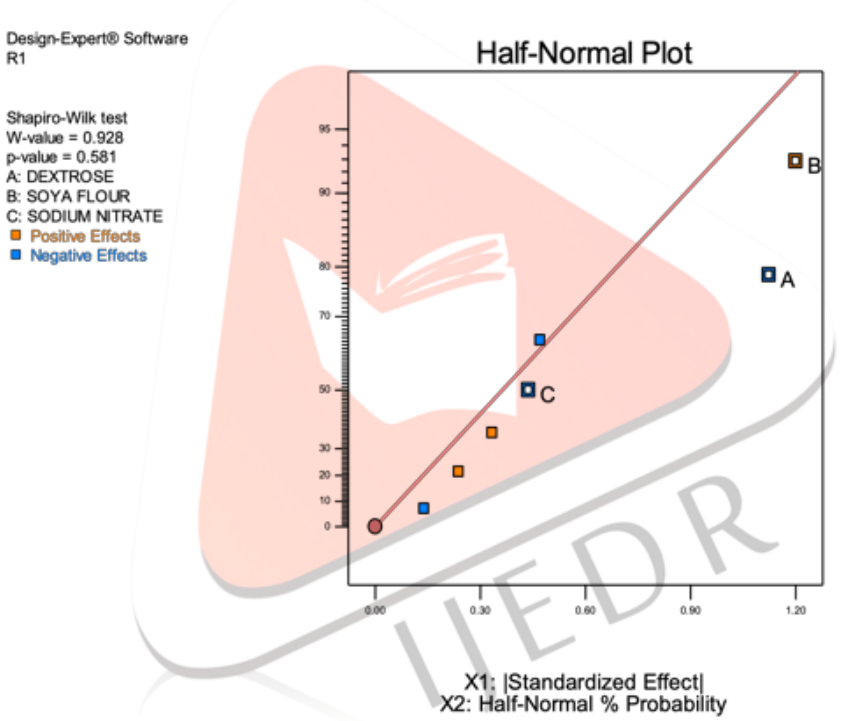
Table 6 shows the response to the various runs

Run	Dextrose	Soya flour	Sodium Nitrate	Yield g/l
1	250	35	12	1.756
2	150	15	12	1.485
3	250	35	4	2.469
4	150	35	4	2.745
5	250	15	12	0.895
6	150	15	12	2.587
7	250	35	4	3.251
8	150	15	4	0.456

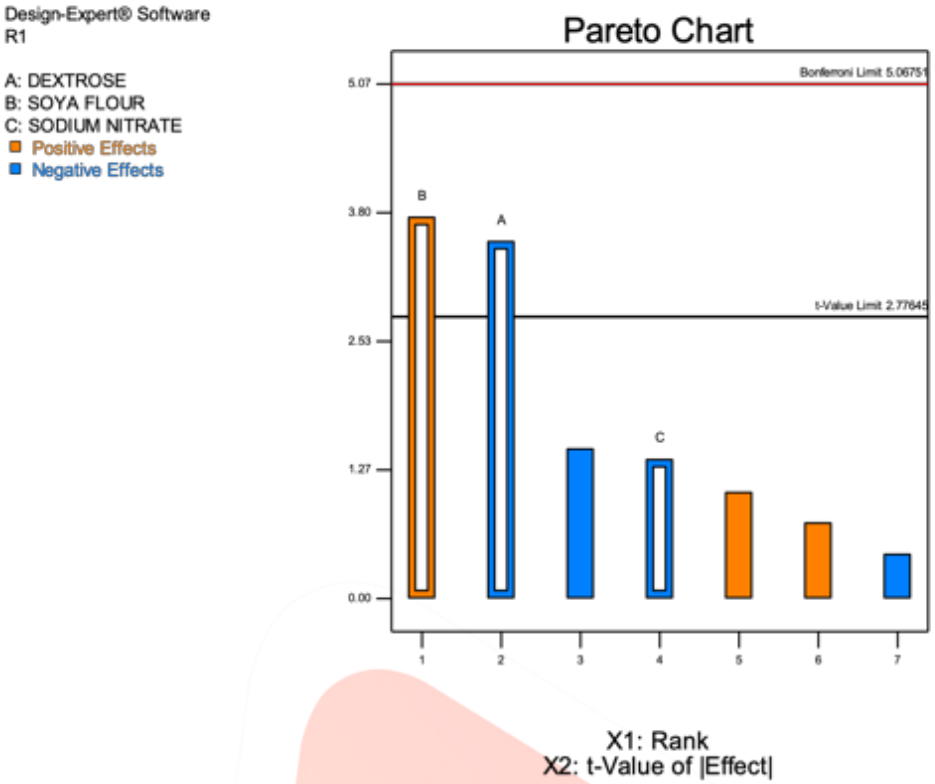
Table 6: Response to the runs

In figure 4, factors (variables) that are at considerable distance from the rest of the points or which are not lying on the line are considered to be significant statistically.

Soya flour is the farthest from the line and is the most significant. The component dextrose and sodium nitrate affects the lincomycin production on the negative scale whereas soya flour affects the production on the positive scale. Thus the concentration of dextrose and soya flour needs to be reduced and the concentration of soya flour need to be increased.



The above data was analyzed using pareto chart (figure 5). According to the chart, soya flour is the most significant component that affects the lincomycin yield on the on the positive scale whereas dextrose is the next component which is significant which affects the yield on the negative scale.



Analysis of significant factors

Analysis was done using Design expert software (Stat-Ease Inc., Version 8.0.7.1)Analysis of Variance (ANOVA) was performed to verify the results obtained through the half and full normal plots of full factorial design. Table 5 summarizes the software generated ANOVA results for the response. In this analysis, the outstanding effects are incorporated into the "model" and the smaller effects are pooled together to estimate the error called "residual". "Cor total" values are the total sum of squares corrected for the mean. It represents the total system variation using the average response as a baseline (Anderson *et al*, 2007).

Source	Sum of Squares	df	Mean Square	F value	p value Prob>F
Model	5.78	3	1.93	9.44	0.0275
A-Dextrose	2.52	1	2.52	12.35	0.0246
B-Soya Flour	2.88	1	2.88	14.09	0.0199
C-Sodium Nitrate	0.38	1	0.38	1.87	0.2432
Residual	0.82	4	0.2		
Cor Total	6.6	7			

Abbreviations df: Degree of Freedom
R-Squared: 0.8762
Pred R Squared: 0.7834
Adeq Precision: 8.637
Table 7: ANOVA analysis

Confidence level was set at 5% and therefore the variables which scored a Probability (P) value less than 0.05 were considered as influential factors affecting the response. Values greater 0.100 indicate that the factors are not significant. The model F value of 9.44 depicts that the model is significant. There is only a 2.75 % chance that a "Model F value" this large could occur due to noise."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The obtained ratio of8.637 indicates an adequate signal. This model can be used to navigate the design space. ANOVA analysis shows that the model is significant, Dextrose and soya flour are significant components as their p value is less than 0.05. Sodium nitrate has a p value of 0.2432 hence it is non significant factor.

Conclusion

Dextrose syrup was optimized as the carbon source best suitable for higher lincomycin production. Soya flour in combination with corn steep liquor were the best organic nitrogen sources. They supported the growth and production to a great extent. Inorganic nitrogen source played a significant role in lincomycin production. Sodium nitrate was chosen as the inorganic

nitrate.Potassium dihydrogen phosphate was the best phosphate source added in the concentration of 0.5g/l. Increased concentration did increase the biomass but had a negative impact on yield. Further the concentration of dextrose syrup, soya flour and sodium nitrate was optimized using full factorial design. According the half normal plot and Pareto chart, the soya flour is the most significant component impacting the lincomycin yield on the positive scale.Dextrose affects the yield on the negative scale. Thus increasing the concentration of soya flour and decreasing the concentration of dextrose would impact have a positive impact on yield.

Media optimization plays a crucial role in the formation, concentration and yield of the end product of fermentation, thus affecting the overall economics.Designing a fermentation media or optimization of fermentation process can be never ending task and every optimization techniques have their own advantages and disadvantages.In the study by combining the one variable at a time and full factorial design we could obtain a 70% rise in the yield of Lincomycin.

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