STUDIES OF VARIOUS PARAMETERS ON L-LYSINE PRODUCTION

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Abstract: Amino acids are the basic bioelements of proteins, which are the most important macromolecules for the functions of humans and animals. Out of the 20 L-amino acids, ecumenically found in most of living organisms, L-lysine is one of the 9 amino acids which are essential for human and animal nutrition. L-lysine is useful as medicament, chemical agent, food material (food industry) and feed additive (animal food). Its demand has been steadily increasing in recent years and several hundred thousands tones of L-lysine (about 800,000 tones/year) are annually produced worldwide almost by microbial fermentation. The objective of this project is to study on L-lysine production by variation of process parameters (temperature and rpm) and variation of fermentation medium composition (carbon and nitrogen source) and effect of biotin by *Corneybacterium glutamicum* in shake flask bioreactor. Stirrer speed, temperature and inoculums medium compositions are varying parameters in Stirred tank bioreactor. . The estimation of Lysine takes places by using Ninhydrin method.

Key words: Lysine, corneybacterium glutamicum, biotin,glucose,ammoniumsulphate.

INTRODUCTION

Out of the twenty naturally occurring amino acids, Lysine (C6H14N2O2; MW 146.19) is one of the 9 essential (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) and commercially important amino acids, ecumenically found in naturally occurring proteins of all living organisms. Its major commercial form is L-Lysine-HCl (L-Lysine monohydrochloride) L- ysine is commonly produced in a stable and non-hygroscopic hydrochlorinated form (H2N(CH2)4 CHNH2CO2H.HCl 2H2O) of a purity higher than 98.5% and moisture content less than 1% [8]. It is mainly used as a feed additive in the animal feed industry, mixed with various common livestock such as cereals which do not contain sufficient levels of L-lysine for the livestock's nutritional requirements, in especially for single-stomach (monogastric) animals like broilers, poultry and swine [6,7,9-12] and as a supplement for humans, improving the feed quality by increasing the absorption of other amino acids. US4327118 [13] describes a preparative method for long lasting solid lysine compositions, suitable for animal feed supplements, which don't agglomerate in the presence of moisture and can for time not necessitating the use of expensive purified L-lysine. As a fine chemical, it is utilized in human medicine, in cosmetics and in the pharmaceutical industry, particularly as ingredients of infusion solutions for pharmaceutical applications [6,14]; and as precursor for industrial chemicals. Furthermore, a production method for industrially producing an optically active lysine derivative useful as a pharmaceutical intermediate is described in [15]. L-lysine can be produced either by a chemical or a biochemical method, which is more economic, even though relatively low yields are obtained during the extraction of Llysine, requiring specific installations and the use of expensive products [13]. The stereospecificity of amino acids and the steadily increasing L-lysine demand necessitates indispensably their fermentative production (the L isomer) over synthetic processes [6,16]. Thus, L-lysineproducing strains of the gram positive corynebacteria, especially *Corynebacterium glutamicum*, *Brevibacterium flavum* and *Brevibacterium lactofermentum*, have been used for the last fifty years for the industrial production of amino acids.

The objectives of study were

To study the l-lysine production by variation of different process parameters (rpm & temperature) and fermentation compositions (carbon source and nitrogen source).
To study the effect of biotin on lysine fermentation process in shake flask bioreactor.

MATERIAL AND METHODS

The microorganism used for the lysine production was *corynebacterium glutamicium*. A complete with following compositions as beef extract 1.0g,bactopeptone 1.0g, glucose 2.0g, Nacl 0.25g, and agar 2.0g per 100ml distilled water and basal medium with composition of kH_2PO_4 1.0 g, $MgSO_4$ .7 H_2O 0.4g, $MnSO_4$. H_2O 2.0 g, $FeSO_4.7H_2O$ 2.0mg, $CaCo_3$ 50g, Glucose 20 g, $(NH_4)_2SO_4$ 10 g,per 1 liter of Distill water was used for the main culture. The specifications for fermentation are Shakeflask250ml, Bioreactorspectrochem, 2L working volume and 3L total volume, glass round bottle reactor. Chemical analytical grade. Spectrophotometer elico mode.

ESTIMATION OF AMINO ACIDS BY NINHYDRIN METHOD

Principle: α – amino acid reacts with ninhydrin a powerful oxidizing agent to from reduce ninhydrin amino acid and water. The formed amino acid and water undergoes oxidative decarboxylation, Co_2, NH_3, aldehyde with one carbon less then the parent amino acid. The formed NH_3 & Nindydrin react with 2 mole of ninhydrin to form a purple colored complex called "Ruchmanns" purple which has absorption maximum 570nm. Amines other than the 2 – amino acid also but react with Ninhydrin forming blue color but with out evolving Co_2 (evolution of Co_2 indication of α – amino acid). How ever proline with 2 amino acid group react with its free amino group to form characteristic brown products.

PREPARATION OF REAGENTS

Ninhydrin reagent –

Solution A: 160 mg of stannous chloride dissolved in few ml of acetate buffer and the total volume was made up to 100 ml with some buffer.
Solution B: 2 gms of Ninhydrin was dissolved in 100 ml of methyl cello solve (or) acetone (or) distilled water.
Reagent: Equal volumes of sol A & sol B were mixed and used as ninhydrin reagent.
Acetate Buffer (0.2M, pH – 5.5)
1.17 ml of CH_3COOH was dissolved in 100ml of distilled water, Solution of Sodium acetate (0.2M):1.64 gms of sodium acetate were dissolved in 100 ml of distill water.
Buffer solvents: Equal volumes of distilled water and n-proponal were mixed and used as a diluent solvent.
Lysine stock standard: 100 mg of lysine was dissolve in 100 ml of distilled water.
Lysine working standard: 10 ml of stock was diluted to 100ml and with distilled water.

PROCEDURE

Blank: In to a clear test tube 1 ml of distilled water 1 mole of ninhydrin reagent were taken and kept in boiling water bath for 15 min. After cooling 5 ml of dilutent solvent was added. This was used to set the absorbance of spectrophotometer to zero at 550nm
Standards: In to series of five test tubes 0.2 – 1 lysine working stranded solution was taken and the total volume has made up to 1ml with distilled water. 1 ml of ninhydrin reagent was added and the tubes were kept in boiling water both for 15 min. After cooling 5 ml of dilutent solvent were added. The intensity of color developed was read at 550 nm in spectrophotometer against blank.

EXPERIMENTAL STUDIES ON L-LYSINE PRODUCTION

Lysine production under shake flask bioreactor is comes under small-scale preparation in this depending upon requirement process parameter variations are taking place. In this shake flask bioreactor the following parameter variations are taking place.i.e.Variation rpm (revolution per minute) of shake flask bioreactor, Variation of temperature, Variation of inoculums medium, Effect of Carbon source (Glucose) Effect of Nitrogen source (Ammoniumsulphate), Effect of Antibiotics (Biotin).

VARIATION OF RPM

250 ml of fermentation medium with adding of enzymes (*Corneybacterium glutamicum)* was taken in two 500 ml conical flasks and the temperature was maintained at 30^0C. The shaking flask incubator was set at 175 rpm and put in to the chamber for 48 hrs. The reading of lysine production for every 6hrs to end of fermentation time was taken. The same procedure was repeated for 200 and 225 rpm.

VARIATION OF TEMPERATURE

The rpm pf shake flask incubator is fixed at 225 rpm then 250ml of fermentation medium with addition of strains (*Corneybacterium glutamicum)* was taken and shake flask bioreactor chamber was set at the temperature of 28^0C.then the readings of lysine production for every 6hrs up to end of fermentation time was taken. The same was repeated for the 30^0C, 32^0C, 34^0C temperatures.

VARIATION OF GLUCOSE

The rpm of shake flask is maintained at 225 rpm then 250 ml of fermentation medium with addition of glucose was taken into conical flask and set at the temperature of 30^0 C, then the reading of lysine produced for every 6 hr to end of the fermentation time was observed. The same procedure was repeated for the other compositions of glucose. I.e. 2 % (v/v), 5% (v/v), 8% (v/v), 10% (v/v) and 12% (v/v).

EFFECT OF AMMONIUM SULPHATE

The rpm and temperature of the fermentation medium was fixed at 225 rpm and 30^0 C repectively, then the ammonium sulphate was added in to the medium and for every 6 hrs to end of the fermentation time the readings were observed. The procedure is repeated for different composition of ammonium sulphate i.e. 2 % (v/v), 3% (v/v), 4% (v/v).

EFFECT OF BIOTIN

Fermentation medium composition was taken and enzymes, 2µm(v/v) of biotin was added and then lysine formation at 48 hours and for every 6 hrs the readings are noted. This is repeated for 5µm (v/v) and 7µm (v/v) of biotin.

LYSINE PRODUCTION USING BIOREACTOR

Lysine production is taking place high scale in bioreactors compare shaking flask incubator. Amount of Fermentation medium preparation takes place depending upon capacity of bioreactor jar. Generally 3 to 5 lit capacity bioreactors are used in educational institutes for research purposes. The shake flask bioreactor is compared to stirred tank reactor and it is observed that the lysine production is more in stirred tank reactor due to better control systems embedded in it. The following parameters variations are taking lysine production
Variation of stirrer speed (RPM), Variation of Temperature. Variation of Inoculums medium.

VARIATION OF STIRRER SPEED (RPM)

Fermentation medium was taken depending on the capacity bioreactor and add inoculums with stirrer speed 300 rpm and take the reading of lysine formation for every 6hrs in 48 hrs of fermentation time. This procedure is repeated for the 400 and 500 rpm.

VARIATION OF TEMPERATURE

Fermentation medium was taken depending bioreactor capacity by adding of inoculums (*Corneybacterium glutamicum)* and fixed stirrer speed of bioreactor i.e. is 400 rpm while varying in temperature of bioreactor. The lysine produced is estimated after every hrs till the end of fermentation time i.e. 48 hrs and the bioreactor temperature is set at 28^0C and results are noted at same stirrer speed. This is repeated for 30^0C & 32^0C temperatures.

RESULTS & DISCUSSIONS

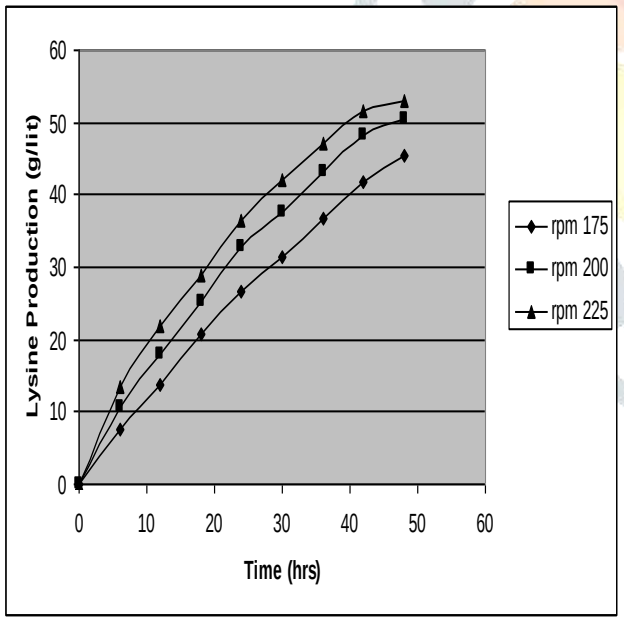
EFFECT OF RPM OF SHAKE FLASK BIOREACTOR

Lysine Production at Different Rpm and At Different Time Intervals

Effect of Rpm on Lysine Production

In shake flask bioreactors the agitation speed varied from 175, 200,and 225 rpm and Lysine production increased rapidly at 175 to 225 rpm, Maximum lysine production is observed at 225 rpm (53 g/lit). Results are shown table 4 and figure 7.This parameter variation shows production L-lysine at different speeds of agitation in shake flask bioreactor. A linear increase in production of lysine is observed with respect to the speed (rotatory shaker) shaking flask bioreactors. This is possibility due to optimum mixing conditions prevailed due to better contact between substrate and microorganism. Specifications: pH 7.5, inoculums 5%, Temperature 30^0C, Glucose composition 7.57 grams in 100 ml distill water.

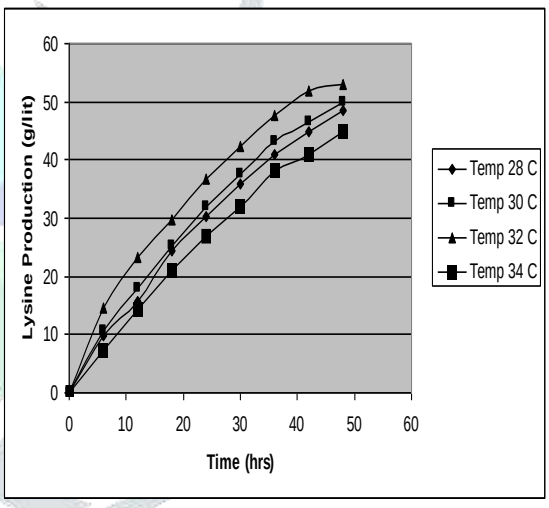
time (hrs)	Lysine Production (g/lit)		
	175 rpm	200 rpm	225 rpm
0	0	0	0
6	7.67	10.58	13.4
12	13.7	17.93	22
18	20.84	25.1	29
24	26.6	32.70	36.4
30	31.35	37.5	42.1
36	36.7	43.1	47
42	41.64	48.35	51.7
48	45.44	50.34	53



EFFECT OF TEMPERATURE

Lysine Production at Different Temperatures

Time (hrs)	Lysine Production (g/lit)			
	28^0C	30^0C	32^0C	34^0C
0	0	0	0	0
6	9.9	10.58	14.5	7.31
12	15.8	17.93	23.32	14.31
18	24.5	25.1	29.7	21
24	30.4	32	36.71	27
30	36	37.5	42.25	32
36	41	43.1	47.75	38
42	45	46.61	51.83	41
48	48.4	50	53	45



Effect of Temperature on production

EFFECT OF TEMPERATURE ON PRODUCTION

The growth rate of microorganisms mainly depending upon process conditions (Temperature) through the reaction sequences make up of the whole metabolism. It is important to reaction shifts in temperature can alter the utilization rate of one component as compared to another thus unbalancing with respect to growth. The early depletion of a critical can shift the culture from balance to unbalanced growth changes its performances. Clearly, for reproducible growth temperature must be rigorously controlled. Temperature variations are taken from 28^0, 30^0, 32^0 and 34^0C lysine and production changes to observe. The maximum lysine production at 32^0C (53 g/lit). Lysine production is decreases above 32^0C. Lysine production was decreased at 34^0C i.e. 45 g/lit this may be due to reduction in microbial preparation. This is in argument with the studies carried out by Hilliger et al (1984). Due to reduce the species formation rate and substrate conversion yield. The results are show in table 5 and figure 8. In Biological process

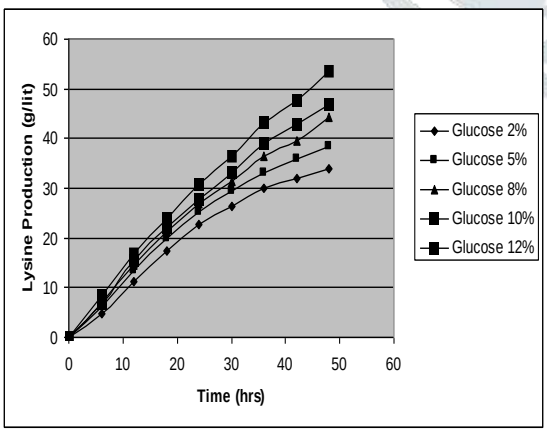
(or) fermentation process temperature variations play a major role and it mainly depend upon microorganism's growths at different temperature. Specifications; pH 7.5 inoculums 5% Rpm 225, glucose 7.57 grams in 100 distill water.

EFFECT OF VARIATION OF GLUCOSE CONCENTRATION

Lysine Production at Different Compositions of Glucose

Time (hrs)	Lysine production (g/lit) with change in Glucose				
	2%(v/v)	5%(v/v)	8%(v/v)	10%(v/v)	12%(v/v)
0	0	0	0	0	0
6	4.79	6.1	6.97	8.48	6.74
12	11.2	13.4	14.49	16.7	15.54
18	17.49	19.8	21.35	23.74	22.2
24	22.64	25.1	26.94	30.8	27.8
30	26.3	29.4	31.45	36.4	33.19
36	29.95	33.1	36.4	43.1	38.9
42	32	36	39.4	47.8	42.85
48	33.8	38.4	44.2	53.5	46.95

Glutamic acid producing bacteria utilize various carbon sources such as glucose, fructose, maltose, ribose or xylose. Glucose concentration was varied in a by study Hirose et al , (1985) and it was found that highest concentration of glucose inhibited growth bacteria and resulted in poor production of Lysine glucose variations are taken from 2% to 12%(v/v) in culture medium different flask. It was observed that 10%(v/v) glucose concentration gave high L-lysine production. Above 10%(v/v) glucose decrease in DCW because increased concentration of inhibited the growth of *Corneybacterium glutamicum* produced 10%(v/v) glucose was 53.31 g/lit. The results are shown table and figure.



This Figure Shows Variation Glucose Composition on Lysine Production In Shake Flask Bioreactor.

Specifications:

pH ---- 7.5 ,

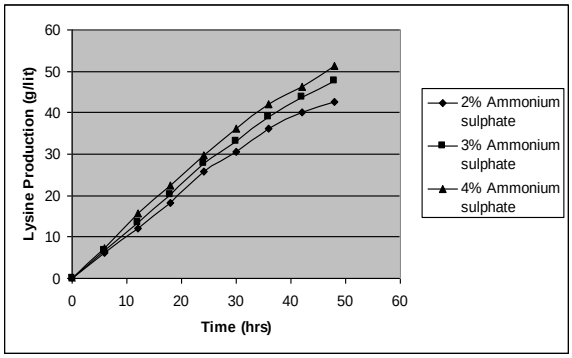
rpm ----225 temperature --- 30^{0}C, inoculum ---- 8%

Effect of variation Ammonium sulphate

Lysine Production at Different Compositions Of Ammonium Sulphate

Time (hrs)	Lysine Production (g/lit) at different compositions of Ammonium sulphate		
	2%(v/v)	3%(v/v)	4%(v/v)
	0	0	0
6	6.2	6.8	7.2
12	12.1	13.4	15.8
18	18.18	20.1	22.4
24	25.9	27.76	29.8
30	30.6	33.1	36.2
36	36.28	38.9	42.1
42	40	43.6	46.4
48	42.53	47.8	51.4

The ample supply of a suitable source is essential for Lysine fermentation, since this contains 19.16% nitrogen. Ammonium salts , such as ammonium chloride is determental to both cell growth and product formation concentration in the medium must be maintained at a low concentration Hirose et al (1985). In this study the effect of different concentration of ammonium sulfate were studied. Maximum lysine (51.4 g.lit) 4% concentration of ammonium sulphate. The DCW increased up to 2.5% ammonium salts due to highest activity of enzymes Pelczar et al (1993). Fe and Mn are important of the trace elements as they play role in the of primary metabolites (Dunn 1985). Keeping in view importance of these elements, the effect of different concentration. The results are shown in table and figure.



Ammonium Sulfate Composition on Lysine Production In Shake Flask Bioreactor.

Specifications: rpm -----225,inoculum 8% temperature 30^0C glucose ----- 10% pH 7.5

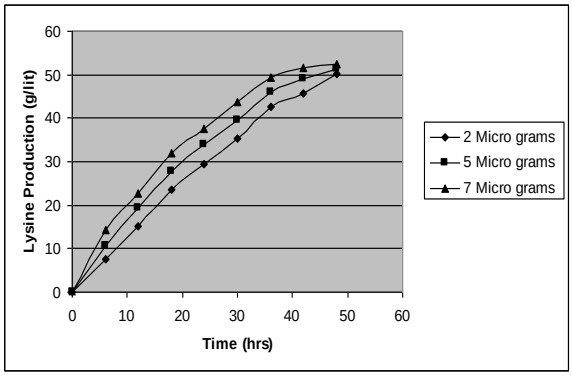
Effect of Biotin

Lysine Production at Different Composition of Biotin

Time (hrs)	Lysine Production (g/lit) at different compositions of biotin		
	2µg(v/v)	5 µg(v/v)	7 µg(v/v)
0	0	0	0
6	7.6	10.79	14.38
12	15.27	19.28	22.64
18	23.54	27.76	32
24	29.57	34	37.59
30	35.36	39.4	43.8
36	42.53	46	49.24
42	45.65	49.2	51.64
48	50.21	51.4	52.46

The result of uptake studies and fatty acid analysis suggested that biotin effected the cell surface, probably the bacterial membrane. It is know that bacteria membrane plays an important role as a charged barrier. This mechanism might also regulate the amount of Lysine released by the cells. Tosaka et .al (1979 a,b) suggested than the effect might be due to the activation of pyruvate carboxylase by biotin. In these investigation the effect of 1 - 30µg 100 ml. The Lysine production has increased when biotin is increased from 2 to 7 micrograms/100 ml of water. The biotin apparently caused some componential changes in cell wall membrane complex allowing in increase in uptake of glucose.

Variation Biotin On Lysine Production In Shake Flask Bioreactor.



Specifications: pH7.5, rpm225, inoculum ---8% , temperature 30^0C, glucose 10%

BIOREACTOR RESULTS:

Effect of Variation of stirrer speed of bioreactor:

Lysine Production at Different Stirrer Speed of Bioreactor (3L)

Time (hrs)	Lysine production (g/lit) at different rpm		
	300 rpm	400 rpm	500 rpm
0	0	0	0
6	9.2	12.54	15.7
12	16.29	19.7	23.4
18	23.4	27.11	30.4
24	30.43	34.60	37.12
30	35.86	39.7	42.94
36	41.71	45.04	49.6
42	47.52	50.84	54.6
48	51.7	54.60	58.76

In case of bioreactor stirrer speed variations are taking place as 300 , 400 and 500 rpm among this stirrer speed highest amount lysine formation taking place at 500 rpm (58.76 g/lit).The results are shown in table and figure .

This is mainly due to better contact of substrate with microorganism at the process conditions take in.

Variation of Stirrer Speed On Lysine Production In Bioreactor

variation of Stirrer Speed on Lysine Production in Bioreactor

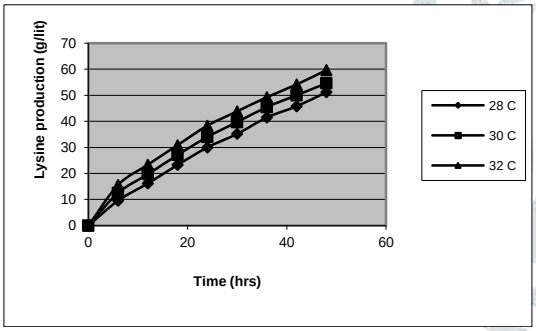
Specifications: temperature 30^0C, glucose 7.57 g per 100 ml distill water, pH 7.5, inoculum - 7%

Variation of Temperature in Stirred tank bioreactor

Lysine Production At Different Temperature Of Stirred Tank Bioreactor

Time (hrs)	Lysine production (g/lit) at temperatures		
	28^0C	30^0C	32^0C
0	0	0	0
6	9.6	12.54	15.6
12	16.2	19.7	23.36
18	23.3	27.1	30.86
24	30	34	38.3
30	35.2	39.7	43.78
36	41.5	45.46	49.21
42	45.8	50	54.1
18	18	19.9	22	24.6
24	21.6	24.7	26.46	30
30	25.1	29.1	32	36.5
36	29.3	33.91	38.2	43.5
42	32	37.77	46	49.7
48	34.2	42.59	51.73	58.46

Variation of temperatures are 28^0C,30^0C and 32^0C from this temperature at 32^0C (59.6 g/lit) highest amount lysine formation take place. The results are shown in table and figure



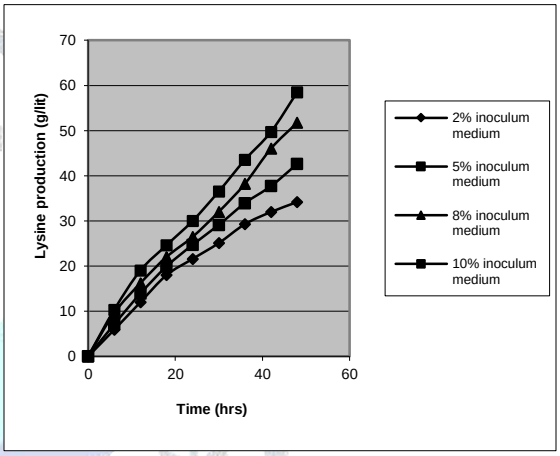
Variation Temperature on Lysine Production in Bioreactor
Specifications: Rpm – 400, glucose 7.57 g per 100 ml distill water, pH ---- 7.5, inoculum ---- 7%

Effect of Variation of inoculum medium compositions

Lysine Production at Different Inoculum Medium Compositions

Time (hrs)	Lysine production (g/lit) at different inoculum medium compositions			
	2%	5%	8%	10%
0	0	0	0	0
6	5.9	7.1	7.6	10.33
12	12	13.9	16.2	19

Variation of Inoculum medium in bioreactor is 2%(v/v), 5%(v/v), 8%(v/v) and 10% (v/v).Highest amount of lysine formation taking place at 10%(v/v)(58.46 g/lit). The results are shown in table and figure.



Inoculum Medium Variation on Lysine Production In Bioreactor.

Specifications: temperature 30^0 C, glucose 7.57 g per 100 ml distill water,pH 7.5, rpm 400

CONCLUSIONS:

The present studies gave good results of production of lysine with *Corneybacterium glutamicum*

Stirred Tank Bioreactor:

By variation of different parameters in stirred tank bioreactor the high amount of lysine production is takes place as follows by varying, rpm, temperature, inoculum composition. By variation of rpm of stirred tank bioreactor at 500 rpm (58.7 g/lit) high amount of lysine production is obtained. By variation of temperature at 32^0C (59.6 g/lit) high amount lysine production is obtained. By variation of inoculum medium at 10%(v/v) (58.4 g/lit) high amount of lysine production is obtained.

The future scope of this project is vary the inoculum compositions 8%, 10%,12% and justify maximum lysine

production. To change carbon & nitrogen sources in medium compositions observe lysine production variations. Finally increase the rpm variations in lysine production shake flaks bioreactor and stirred tank bioreactor.

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