To develop a Functional Food formulation comprising of *Saccharomyces cerevisiae* and *Spirulina* and to study the effect of fermentation conditions on the protein content of SCP from *Saccharomyces cerevisiae.*

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Submitted in partial fulfillment of the Degree of Bachelors' of Technology

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS,
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CERTIFICATE

This is to certify that the work entitled, "**To develop a Functional Food formulation comprising of *Saccharomyces cerevisiae* and *Spirulina* and study the effect of fermentation conditions on the protein of SCP from *Saccharomyces cerevisiae*.**" submitted by Varun Chowdhary and Nawanshu Chhabra in partial fulfillment for the award of degree of Bachelor of Technology in BIOTECHNOLOGY of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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With utmost respect, pride and gratitude we would like to thank Dr Gargi Dey for her esteemed guidance and intellectual stimuli in enabling us to do this project honorably and scientifically.

We would also like to thank Dr. S. Ramachandran of BITS, Pilani, Dr. Suresh Walia and Dr. Dolly W. Dhar of I.A.R.I, Pusa for their expert guidance and magnanimity in providing us with strains of *Spirulina.*

We would also like to thank all the PhD scholars who with their timely help: materials, intellectual and psychological, were always there when we needed them.

Varun Chowdhary (051562)
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Abstract

Baker's Yeast - The production of baker's yeast is the largest domestic use of a microorganism for food purposes. Baker's yeast is a strain of *Saccharomyces cerevisiae.* The strain of the yeast is carefully selected for its capacity to produce abundant gas quickly, its viability during ordinary storage, and its ability to produce desirable flavor. Yeasts have been the most important group of microorganisms exploited by mankind. Except the diverse traditional using in human and animal nutrition, yeast is finding importance as important biological agents for biotransformations. Studies have shown that these components present in yeast have potential health benefits such as, improved immune response, reduction of cholesterol, and anti-cancer properties.

Spirulina is the common name for human and animal food supplements produced primarily from two species of cyanobacteria (also known as blue-green algae): *Arthrospira platensis*, and *Arthrospira maxima.* These and other *Arthrospira* species were once classified in the genus Spirulina. There is now agreement that they are distinct genera, and that the food species belong to *Arthrospira*; nonetheless, the older term *Spirulina* remains the popular name. Spirulina is cultivated around the world, and is used as a human dietary supplement as well as a whole food and is available in tablet, flake, and powder form. It is also used as a feed supplement in the aquaculture, aquarium, and poultry industries.

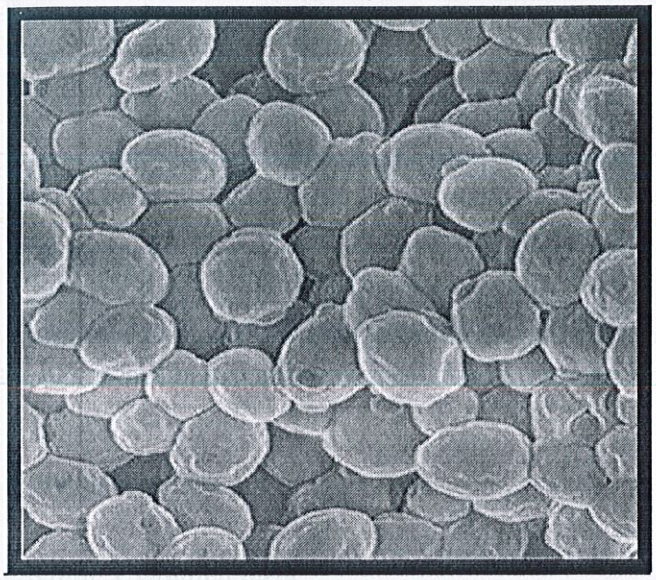
Spirulina is effective for the clinical improvement of melanosis and keratosis due to chronic arsenic poisoning; improves weight-gain and corrects anemia in both HIV-infected and HIV-negative undernourished children; and protects against hay fever.

The project is to develop a functional food derived from Yeast and Spirulina and along the course of the project we conclude that the best nitrogen source for growing yeast is ammonium sulfate and it is best suited to the 120 rpm.

The large amount of sugars present in the molasses, to the amount of about 50% is essential for the production of CO_2 and the stationary cultures of Yeast showed a lot of CO_2 evolution which augmented the growth of Spirulina in cultures which were coupled with the yeast.

Chapter: 1
INTRODUCTION

1.1 *Saccharomyces cerevisiae*



Yeast:courtesy www.rug.nl/.../cio/student-projects/yeast.jpg

Domain: Eukaryota

Kingdom: Fungi

Phylum: Ascomycota

Class: Saccharomycetes

Genus: Saccharomyces

Species: cerevisiae

Common name: Baker's Yeast

1.2 *Spirulina*

Genus: (Illustrations of The Japanese Fresh-water Algae, 1977).

Domain: Bacteria

Phylum: Cyanobacteria

Class: Chroobacteria

Order:Oscillatoriales

Family: Phormidiacea

Genus: Arthrospira(Spirulina)

Species: platensis

Common name: *Spirulina*

The aim of this project is to develop a functional food formulation comprising of yeast a fungus with wide applications in the food industry and also is one of the most widely used microbial system in the whole world with wide applications that include baking, brewing and consumption as an SCP.

And Spirulina which is one of the most nutritious SCP on the planet an alga it grows in open ponds in the tropical countries primarily in South America and Africa. Its nutrition profile is extensive with protein being the major component and also some micronutrients which are also very essential.

The project aims at creating a process in which there is optimum production of yeast and Spirulina in a combined system.

Nutrient Profile:-

Saccharomyces cerevisiae (per 100g -www.nutritiondata.com)

Components	*Amount/100g*
Total fat	1g
Saturated fat	0g
Cholesterol	-
Sodium	5 mg
Total Carbohydrate	5 g
Dietary fiber	4 g
Sugars	0 g
Protein	48 g

- **An excellent source of protein (52%), containing essential amino acids.**
- **Rich in vitamins, especially the B-complex vitamins.**
- **An excellent source of folic acid, which is important for formation, growth, and regenertion of red blood cells.**
- **Naturally low in fat and salt.**
- **It contains additional functional and beneficial components such as beta-1,3 glucan, trehalose, mannan and glutathione.**

Nutrient profile:-

Spirulina (per 100g -www.nutritiondata.com)

Component	*Amount/100g*
Calories	346
Fats	6.7 g
Protein	64.6 g
Carbohydrates	16.1 g
Crude fibre	9.3 g
Vitamins	B carotene, biotin, cyanocobalamine, folic acid, riboflavin, thiamine, tocopherol
Minerals	Calcium, phosphorous, iron, sodium, potassium.
Essential amino acids	Lysine, cysteine, methionine, phenylalanine, threonine.

Complementary nutrients

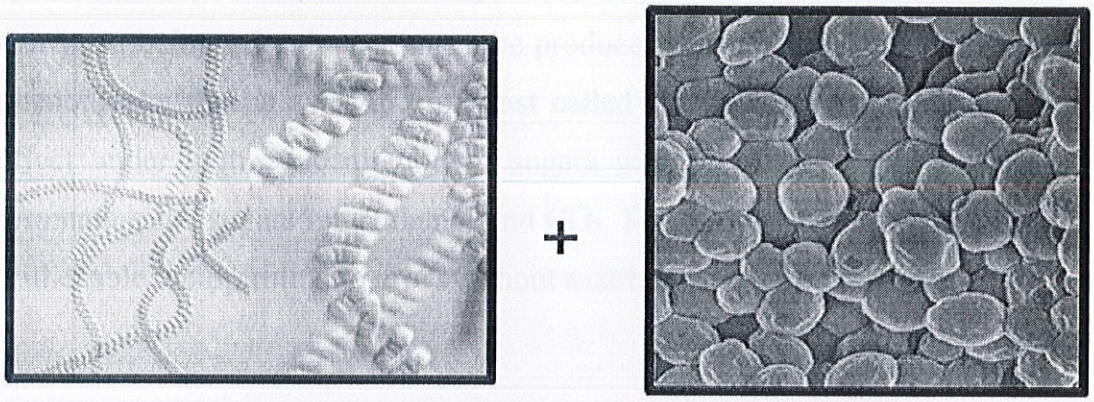
- **Beta-1, 3 glucan, trehalose, mannan and glutathione. Abundant in yeast but not in Spirulina.**
- **B-complex vitamins, folic acid abundant in yeast but not in Spirulina.**
- **Lysine, cysteine, methionine, phenylalanine, threonine amino acids found in Spirulina.**
- **These are the nutrients which are complementary to each other i.e present in abundance in one but not in the other. Present in Spirulina but not in yeast.**

The project is to formulate a functional food that has both yeast and Spirulina and also thus their respective nutrient profile which are naturally complementary to each other thus forming a functional food which is complete in almost all respects.

Objective:

Our objective in this project is of a dual nature that is our project has a dual aim.

1. **Product development:** To develop a Functional food (nutraceutical) from *Spirulina* and yeast and to study the effects of different fermentation conditions on the protein and carbohydrate profile of yeast.



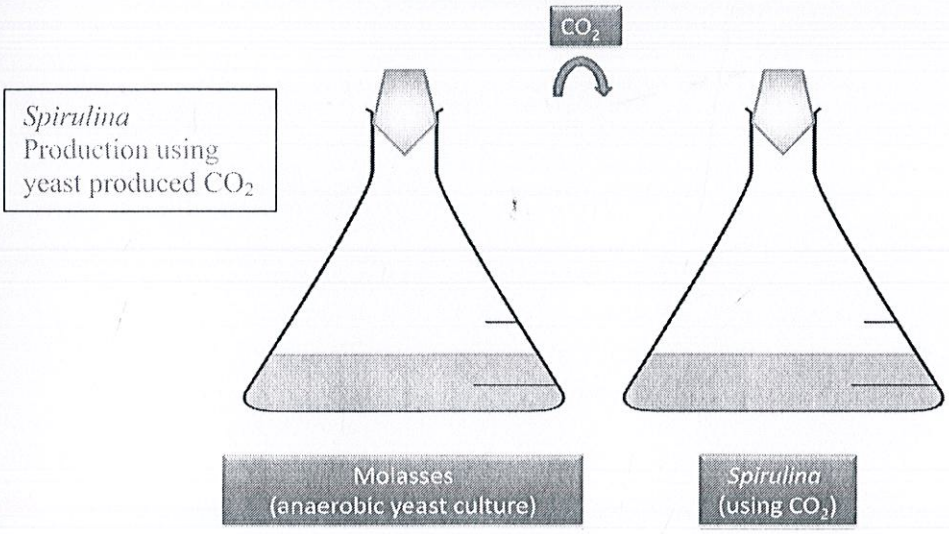
Spirulina + Yeast

= Functional food

This part of our project is to develop a functional food (nutraceutical) from the yeast and *Spirulina* biomass that is produced by the unification process and also to characterize the carbohydrate and protein profile of the SCP obtained from the yeast. This is a naturally suitable choice because of the fact that these two have a complementary nutrient profile and together the best combination available and also can grow naturally with each other in a unified process. In today's hectic and stressful lifestyle this combination can target many of the lifestyle diseases like Hypercholestremia, diabetes, strokes, anemia.

Process development:-

This module of the project aims at developing a unified process for the production of *Spirulina* and baker's yeast biomass that can be feasible on an industrial scale and that can produce a viable amount of both yeast and *Spirulina*. This has been thought of as possible because of the fact that *Spirulina* being a cyanobacterium can utilize Carbon dioxide as a carbon source and grows effectively only requiring a nitrogen source. This is the salient feature of *Spirulina* that has made its pairing with yeast as effective because of the fact that yeast being a facultative anaerobe produces Ethanol and CO_2. But a certain phenomenon that is a feature of yeast called the Crabtree effect. Due to this effect under high concentration of sugars yeast don't grow but rather start fermenting the sugars into Ethanol and CO_2. This production of carbon dioxide will enable the Spirulina to grow without a carbon source in the media.



Advantage of the process:-

1. **The main advantage of the process is that we are combining two naturally complementary substances in a unified process.**

2. **The whole process from an industrial point of view also gives another important by product i.e. Ethanol.**

Chapter 2
Materials & Methods

Materials:-

- Baker's Yeast: *Saccharomyces cerevisae.* Dried form that is commercially available. To be used for CO_2 production and as a nutraceutical.
- *Spirulina*: *Arthrospira/Spirulina platensis* a well known Dieatary supplement. Cultures SP1 and SP2 from BITS, Pilani and IARI, Pusa, New Delhi.
- Molasses: A cheap carbon source ideal for industrial scale.
- Inorganic nitrogen sources: Ammonium Nitrate, Ammonium Sulfate, Ammonium Phosphate.
- YPD media: 1% yeast extract, 2% Peptone, 2% Dextrose. Used for the revival of the dried yeast cultures and for the preparation of the inoculums.
- Zarrouk's media: Basic inorganic media for *Spirulina.*
- CFTRI media : Alternative media for *Spirulina.*
- Reducing sugar estimation: DNS reagent test.
- Protein estimation: Lowry's method.
- Spectrophotometer: Required at different wavelength settings as the experiment states.

Methods:-

Yeast

- Standardization of molasses (Carbon source) concentration.
- Standardization of Nitrogen source of media.
- Standardization of optimum rpm.
- Standardization of biomass yield at anaerobic and partially anaerobic yeast growth media.
- To estimate the biomass yield of all media.
- Estimation of reducing sugar content of molasses.
- Estimation of protein content of yeast.

Spirulina

- Basic growth and culture techniques.
- Unification of the Spirulina and the yeast and the quantification of the biomass.

Pre-inoculum:

- 100 ml of ypd media made and autoclaved.
- 0.5 gms of dried yeast is inoculated in the media.
- This media is kept in the aerobic conditions i.e. Shaker incubator for 24 hrs.
- Temperature is maintained at 30.0 ^{0} C.
- Rpm is maintained at 120.

Inoculum:

- 1.5 ml of the pre-inoculum is inoculated into a fresh 100 ml ypd media.
- This is kept at the same conditions as the pre inoculums for 24 hrs.
- This is done to revive the yeast completely.

Preparation of media:

- The media for the growth of the yeast is molasses as the carbon source and an inorganic nitrogen source.
- Different concentrations of molasses is taken and mixed with distilled water to get appropriate concentrations.
- Inorganic nitrogen source is added in the amount 0.1 gm/100 ml of media.
- This inorganic source is varied and different compounds are tried for the same molasses concentrations.
- 1.5 ml of the inoculums media is added and then this molasses is set up for 48 hrs in the same conditions as the pre-inoculum and inoculum.

Completely Anaerobic Cultures:

- Optimum molasses concentration with inorganic nitrogen source is taken and kept in anaerobic conditions (Incubator) at 30.0^0 C.

Estimation of Biomass:

- The 48 hr culture is taken and centrifuged in pre weighed centrifuge tubes for 10 minutes at 6000 rpm.
- The supernatant is discarded and the pellet is washed with water and then centrifuged again at 6000 rpm for 10 minutes.
- This biomass is weighed and this is the wet biomass.
- This gives us the result of a biomass experiment.

***Spirulina* growth:**

- The media used for culturing *Spirulina* is Zarrouk's media-18.0 g $NaHCO_3$, 2.5 g $NaNO_3$, 0.5 g K_2HPO_4, 1.0 g K_2SO_4, 1.0 g NaCl,

0.04 g CaCl2, 0.08 g Na2 EDTA, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$ and 1.0 ml trace elements (TE). TE (g/L): H_3BO_3 2.86; $(NH_4)_6Mo7O24$ 0.02; $MnCl_2 \cdot 4H_2O$ 1.8; Cu_2SO_4 0.08; $ZnSO_4 \cdot 7H_2O$ 0.22.

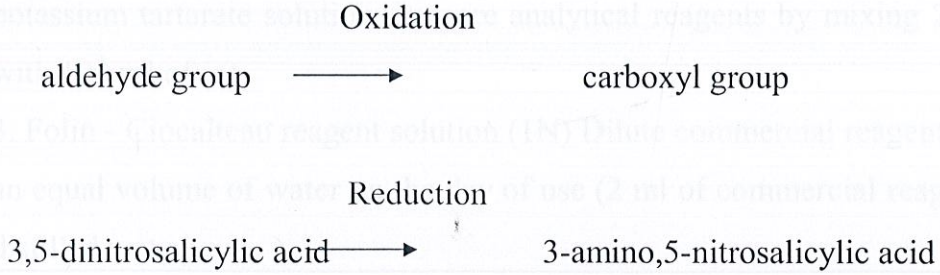
- The process takes about 8-10 days at 23-27 °C, pH maintained at9.5-10.

The aim is to also produce the Spirulina biomass along with the yeast biomass.

Reducing sugar estimation:

DNS reagent test

This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions:



The above reaction scheme shows that one mole of sugar will react with one mole of 3,5-dinitrosalicylic acid. Different reducing sugars generally yield different color intensities; thus, it is necessary to calibrate for each sugar. Although this is a convenient and relatively inexpensive method, due to the relatively low specificity, one must run blanks diligently if the colorimetric results are to be interpreted correctly and accurately.

Procedure

- It was done by using DNS solution (Miller and G.L., 1959). Standard curve for sugar estimation was prepared using dextrose as a standard at different concentration from 0.2 to 15%. 100µl of standard or sample was added to 750µl of DNS solution and incubated at 60°C for 5mins. Optical density was measured at 540nm.

Protein estimation:

Reagents Required

1. BSA stock solution (1mg/ml),
2. Analytical reagents:

(a) 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water.)

(b) 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% sodium potassium tartarate solution. Prepare analytical reagents by mixing 2 ml of (b) with 100 ml of (a).

3. Folin - Ciocalteau reagent solution (1N) Dilute commercial reagent (2N) with an equal volume of water on the day of use (2 ml of commercial reagent + 2 ml distilled water)

Principle

The phenolic group of tyrosine and trytophan residues (amino acid) in a protein will produce a blue purple color complex, with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent which consists of sodium tungstate, molybdate and phosphate. Thus the intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different proteins. Most proteins estimation techniques use Bovin Serum Albumin (BSA) universally as a standard protein, because of its low cost, high

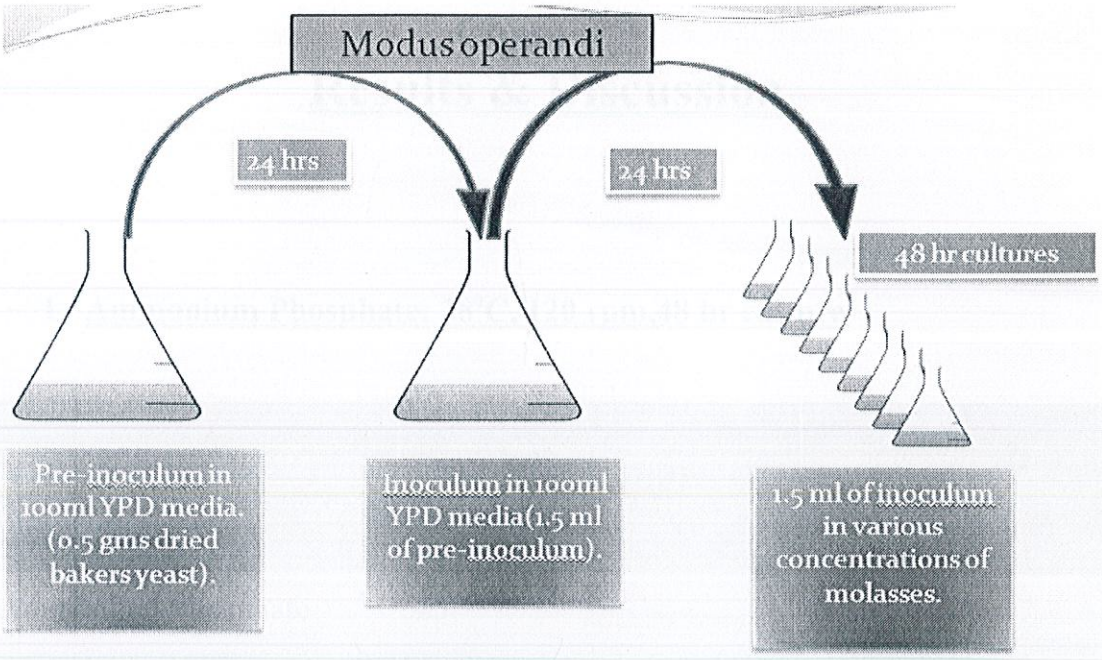
purity and ready availability. The method is sensitive down to about 10 μg/ml and is probably the most widely used protein assay despite its being only a relative method, subject to interference from Tris buffer, EDTA, nonionic and cationic detergents, carbohydrate, lipids and some salts. The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9 to 10.5 is essential.(Lowry 1951)

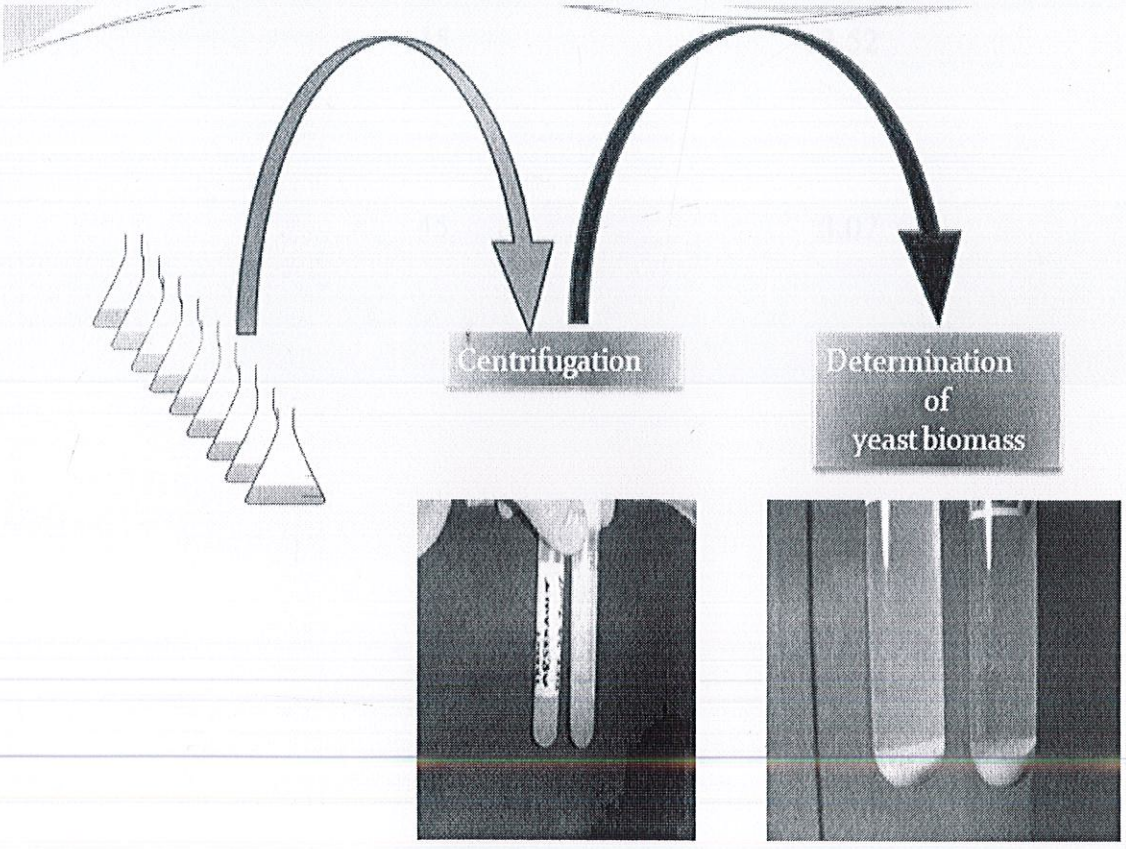
pH change analysis:

- Water and Molasses inoculated with yeast is unified.
- After every 24 Hours pH of the water is measured.
- 5 ml of undiluted molasses is added to the molasses flask after every 24 hours.

Spirulina biomass estimation:

- The Spirulina is unified with the molasses.
- The biomass is estimated by centrifuging the media at 10000 rpm for 10 minutes in a pre-weighed centrifuge tube. The biomass was measured in wet wt form.
- This biomass is then compared to a non unified growth biomass.

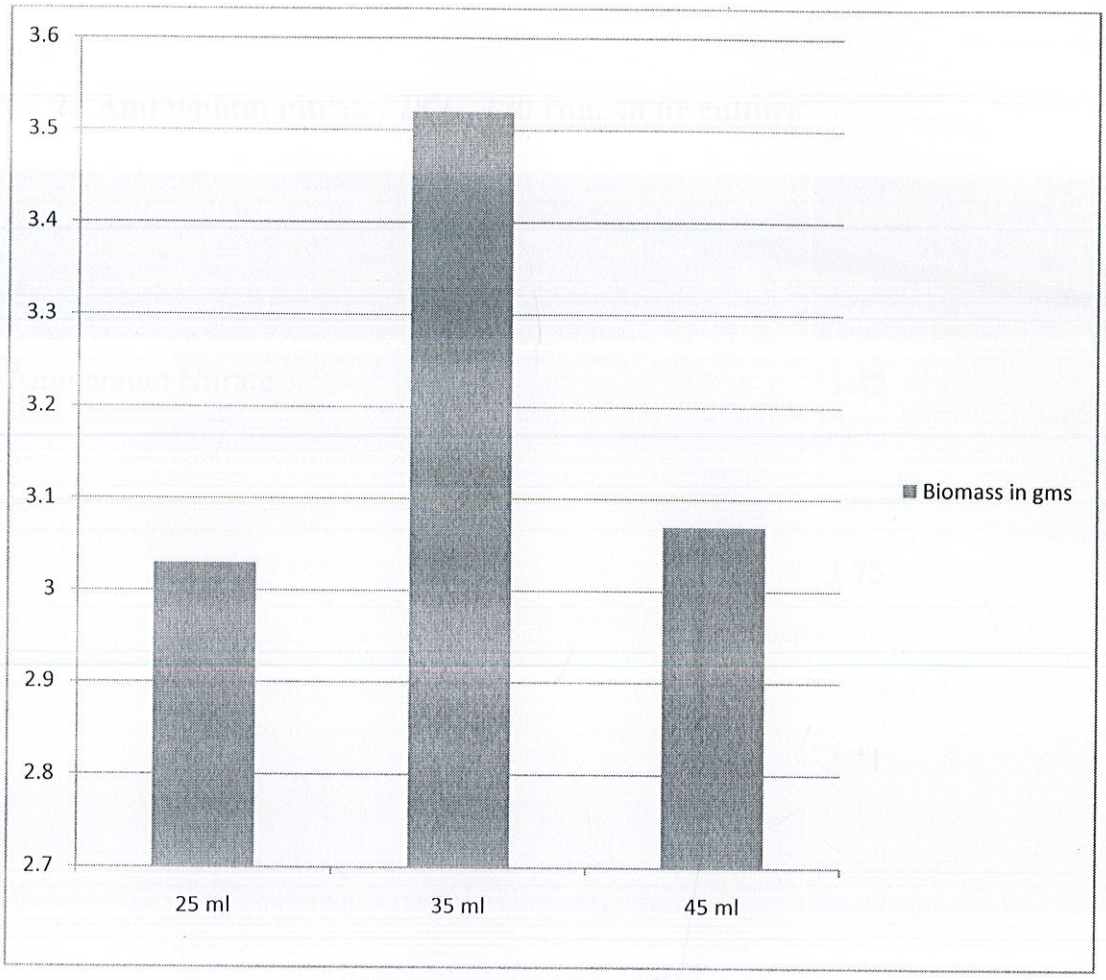

Modus operandi
24 hrs
24 hrs
48 hr cultures
Pre-inoculum in 100ml YPD media. (0.5 gms dried bakers yeast).
Inoculum in 100ml YPD media(1.5 ml of pre-inoculum).
1.5 ml of inoculum in various concentrations of molasses.


Centrifugation
Determination of yeast biomass

Chapter 3
Results & Discussion

1. Ammonium Phosphate: 28°C, 120 rpm,48 hr culture

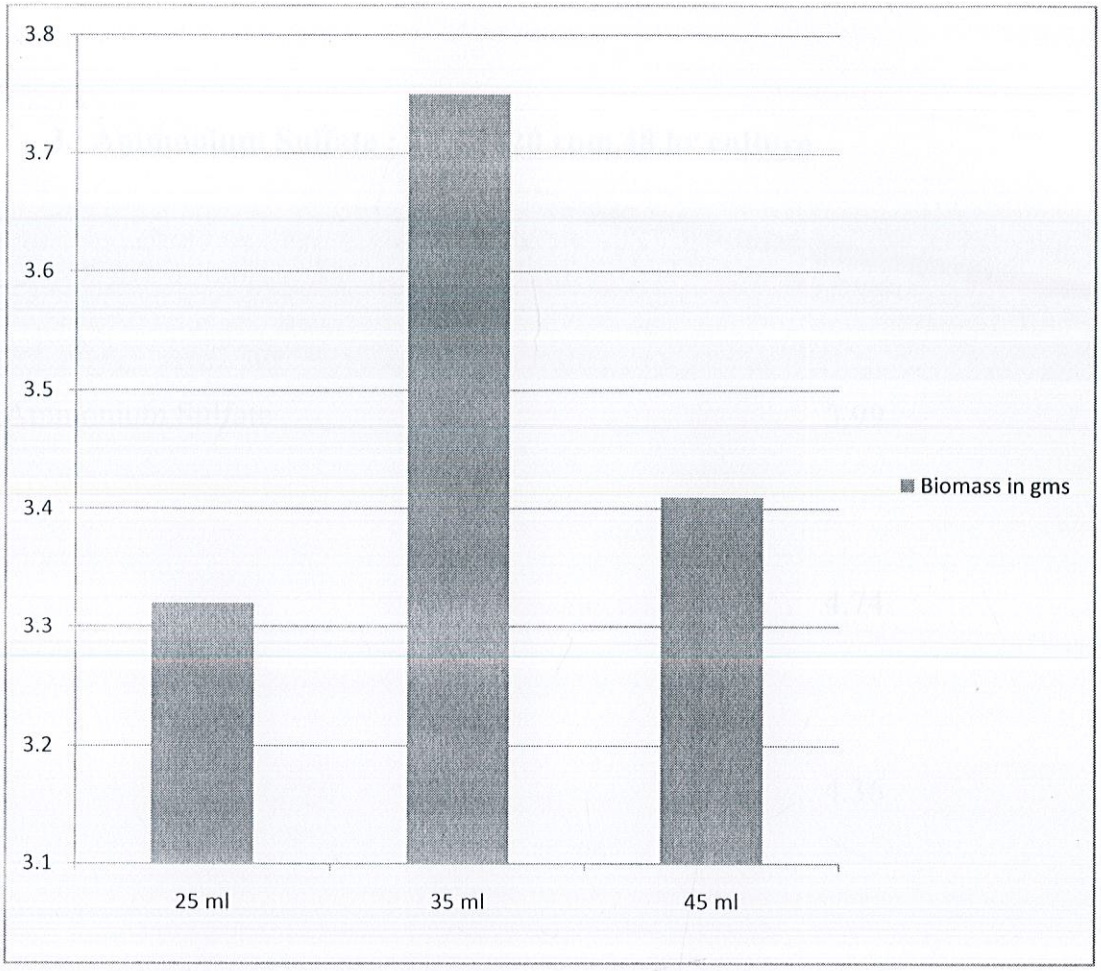
N_2 Source (0.1g/100ml)	Molasses concentration	Yield (in gms) Wet weight
Ammonium Phosphate	25	3.03
	35	3.52
	45	3.07



Molasses Concentration in ml

2. **Ammonium nitrate: 28°C, 120 rpm,48 hr culture**

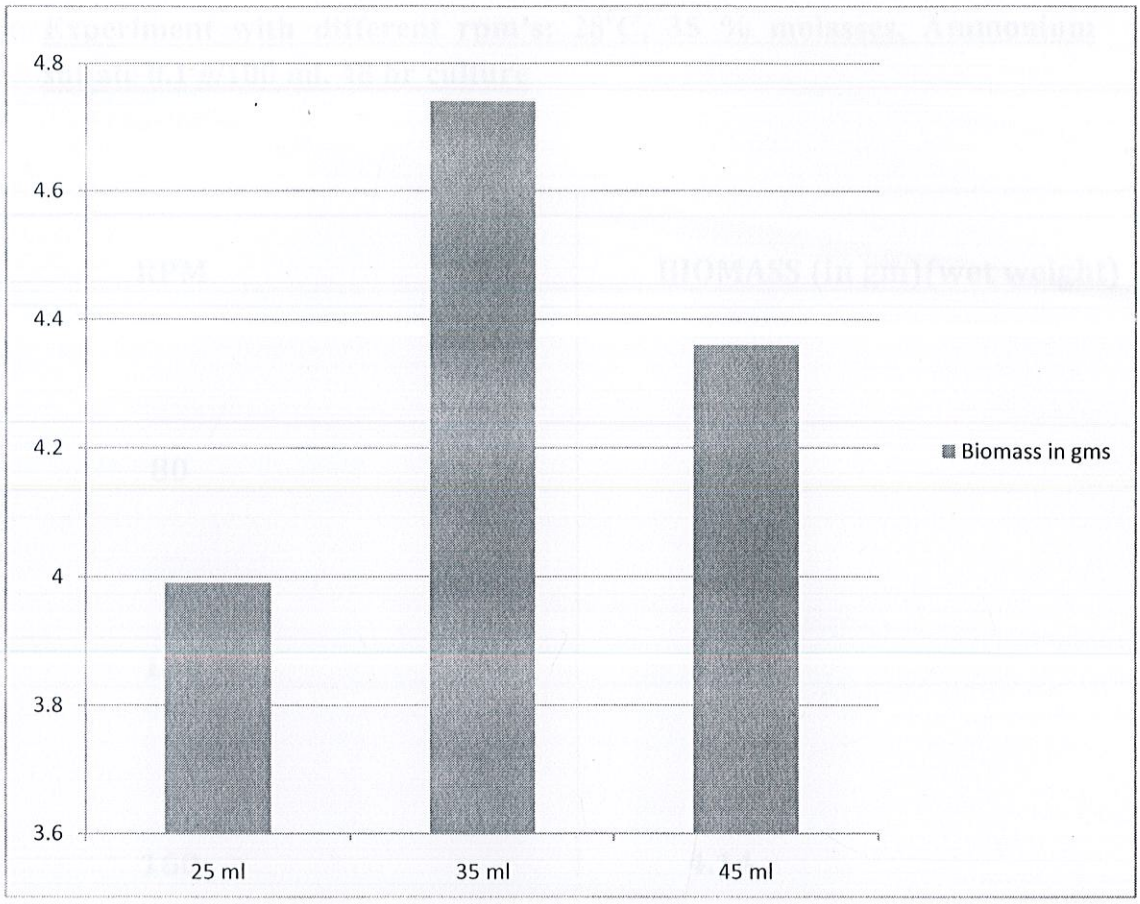
N_2 Source (0.1g/100ml)	Molasses concentration (in ml)	Yield (in gms) Wet weight
Ammonium Nitrate	25	3.32
	35	3.75
	45	3.41



Molasses Concentration in ml

3. **Ammonium Sulfate : 28°C, 120 rpm,48 hr culture**

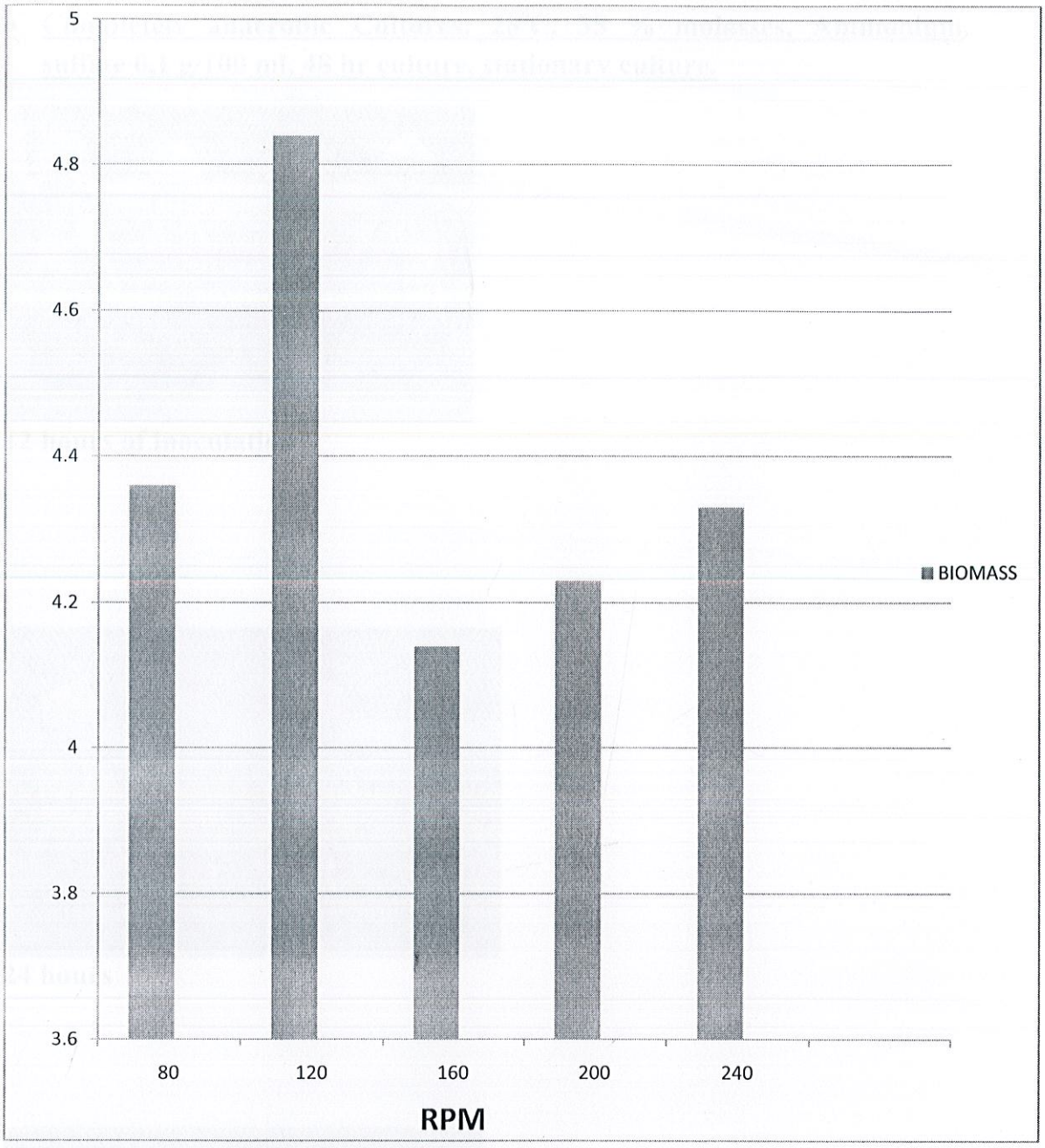
N_2 Source (0.1g/100ml)	Molasses concentration (in ml)	Yield (in gms) Wet weight
Ammonium Sulfate	25	3.99
	35	4.74
	45	4.36



Molasses Concentration in ml

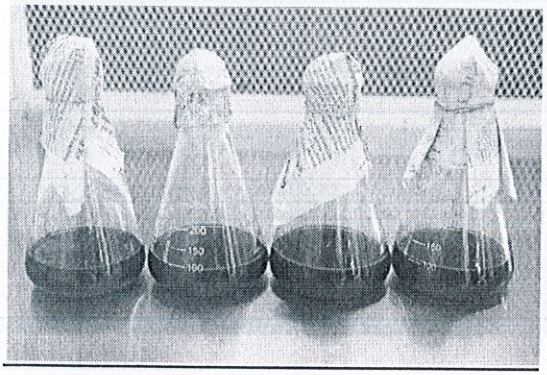
- **Experiment with different rpm's: 28°C, 35 % molasses, Ammonium sulfate 0.1 g/100 ml, 48 hr culture**

RPM	BIOMASS (in gm)(wet weight)
80	4.36
120	4.84
160	4.14
200	4.23
240	4.33

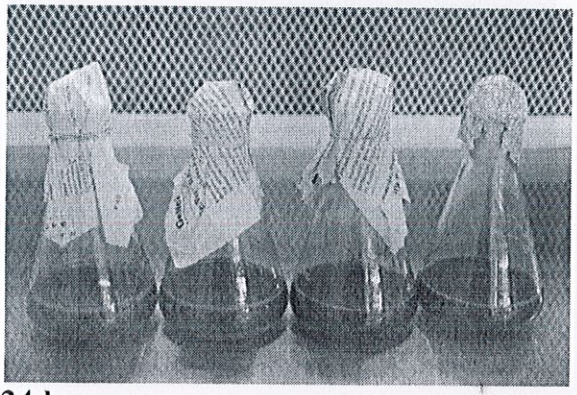


Comparative growth of yeast at various rpm's

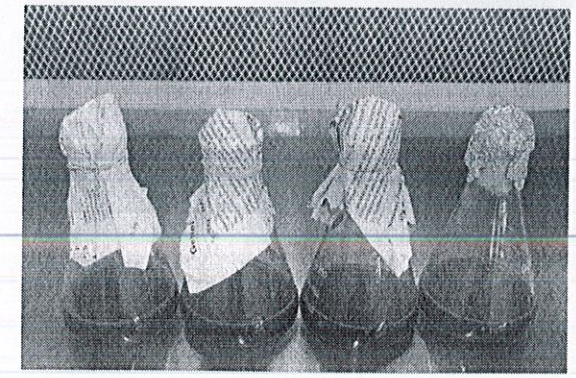
- **Completely anaerobic Cultures: 28°C, 35 % molasses, Ammonium sulfate 0.1 g/100 ml, 48 hr culture, stationary culture.**



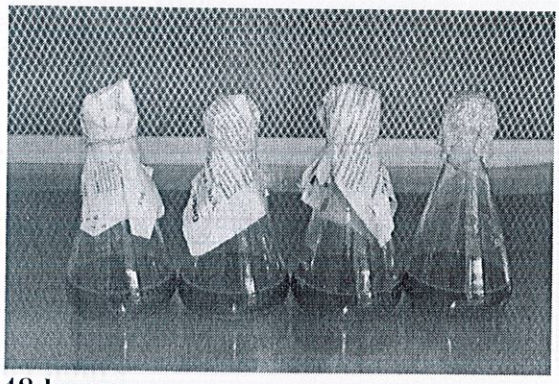
12 hours of inoculation



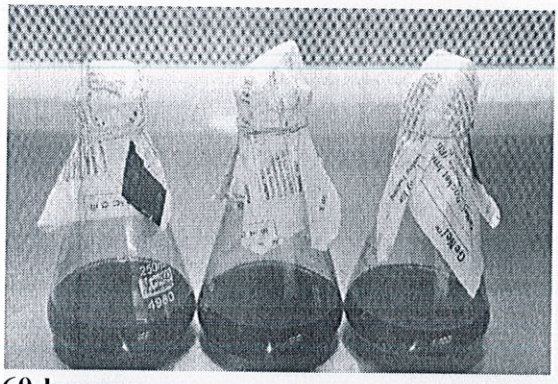
24 hours



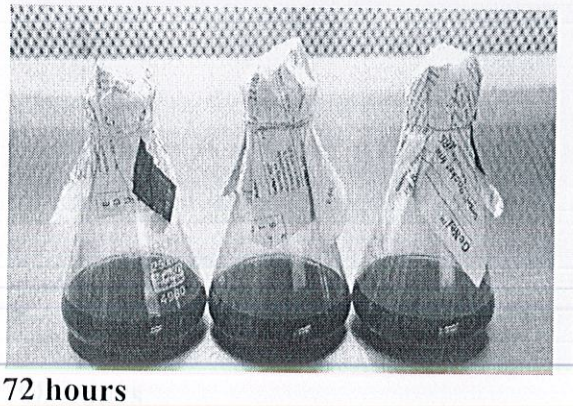
36 hour



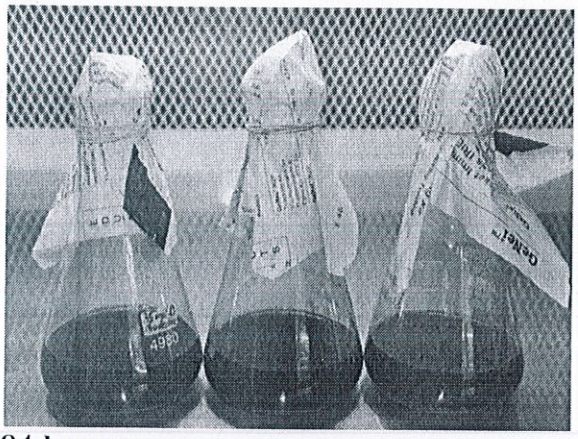
48 hours



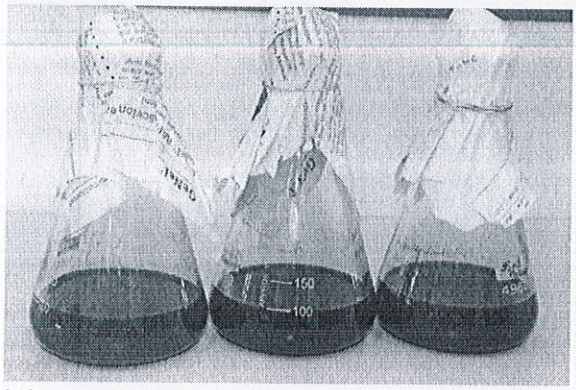
60 hours



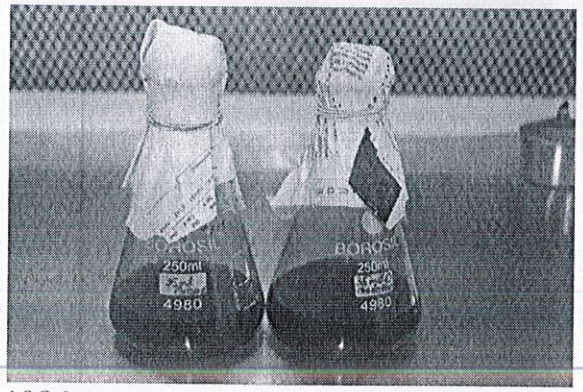
72 hours



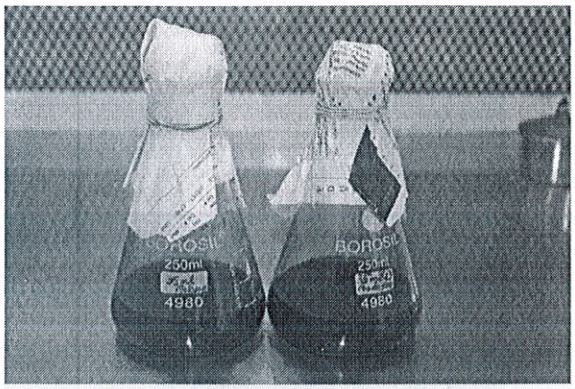
84 hours



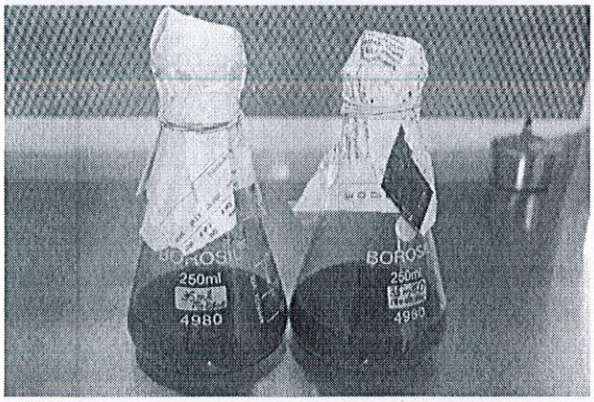
96 hours



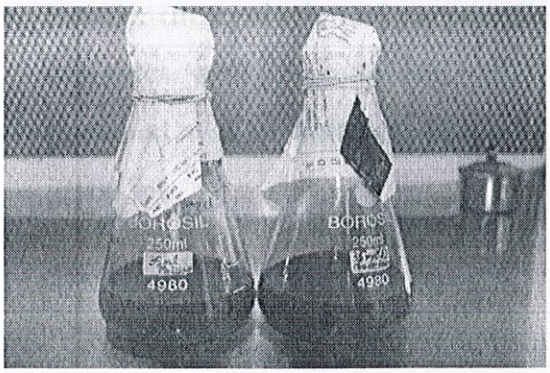
108 hours



120 hours

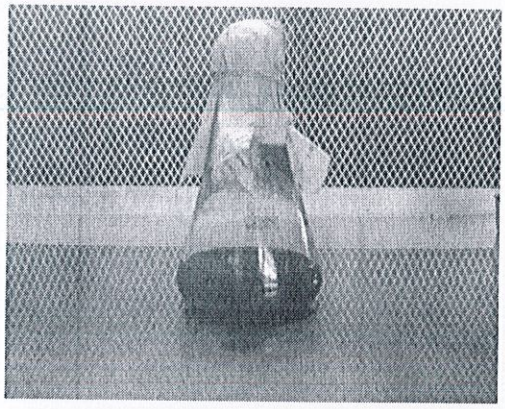


132 hours

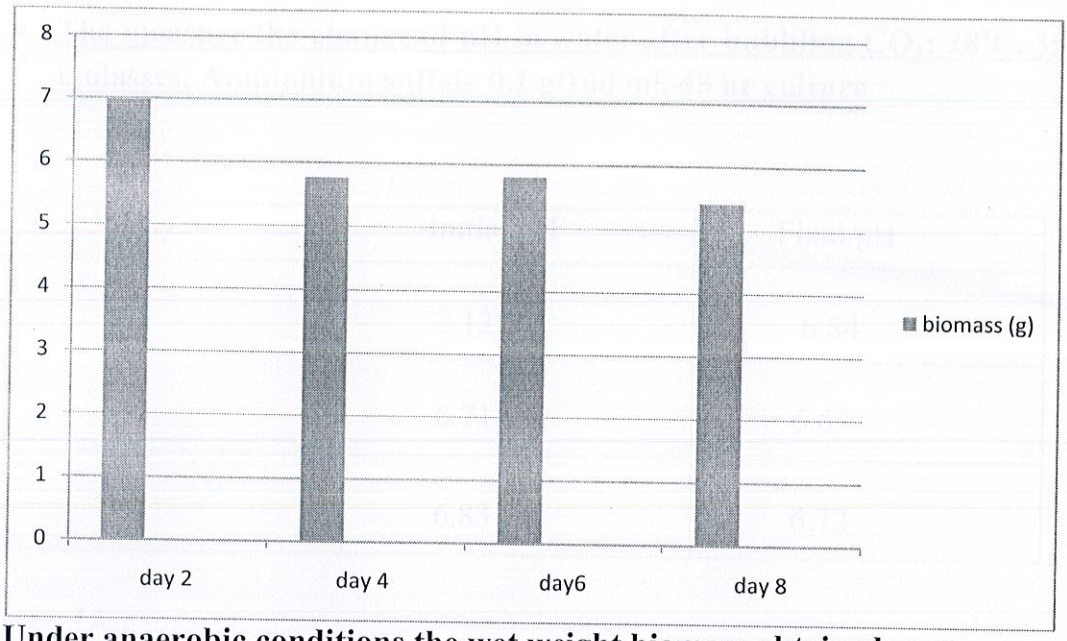


144 hours

7th day, 8th day (below)



- **Result: the evolution of carbon dioxide stopped after 48 hours for 35 ml molasses and ammonium sulfate as the nitrogen source biomass obtained was as follows. 28°C, 35 % molasses, Ammonium sulfate 0.1 g/100 ml.**



Under anaerobic conditions the wet weight biomass obtained was:-

Day2- 6.98 grams

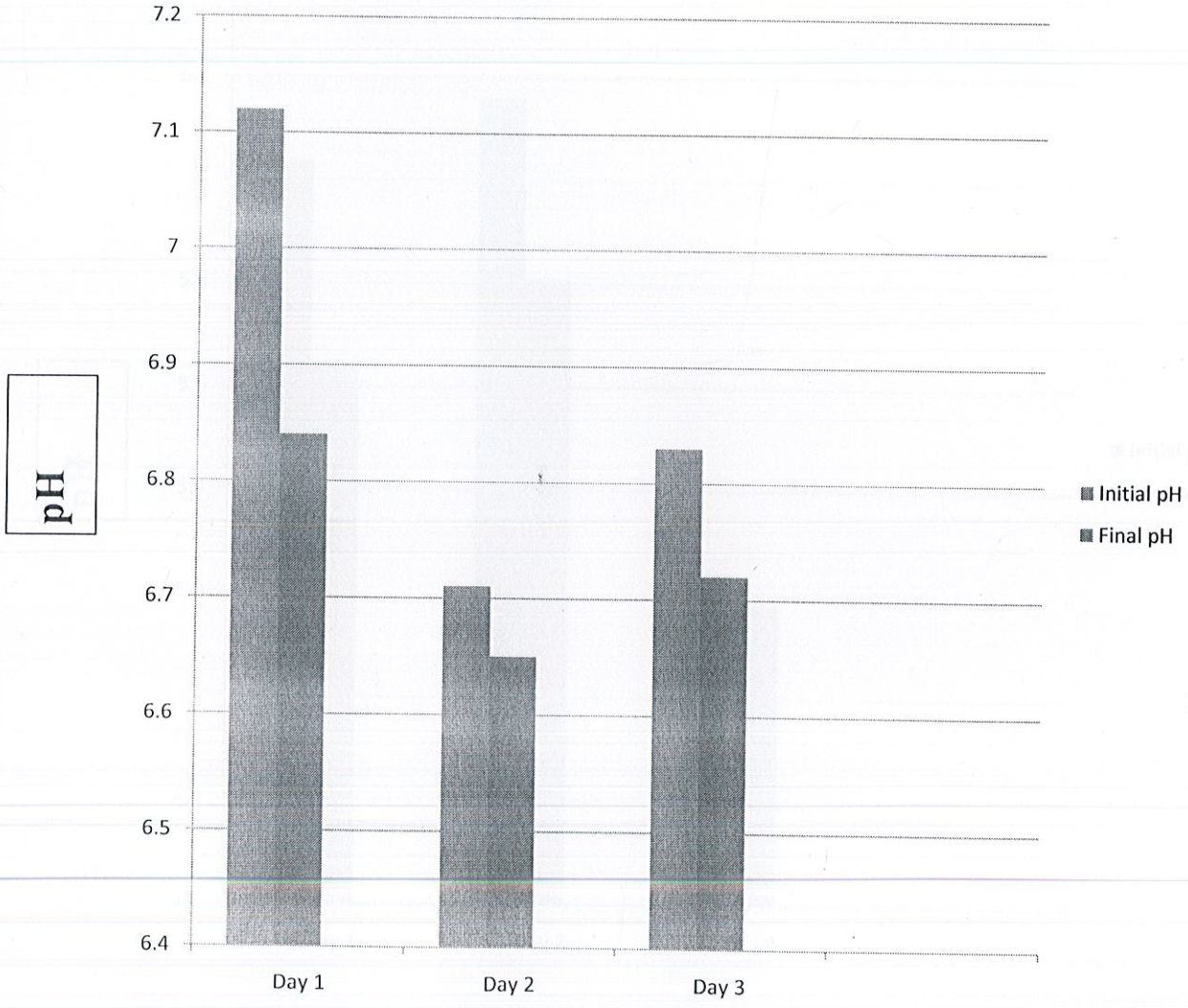
Day4- 5.77 grams

Day6- 5.81 grams

Day8- 5.42 grams

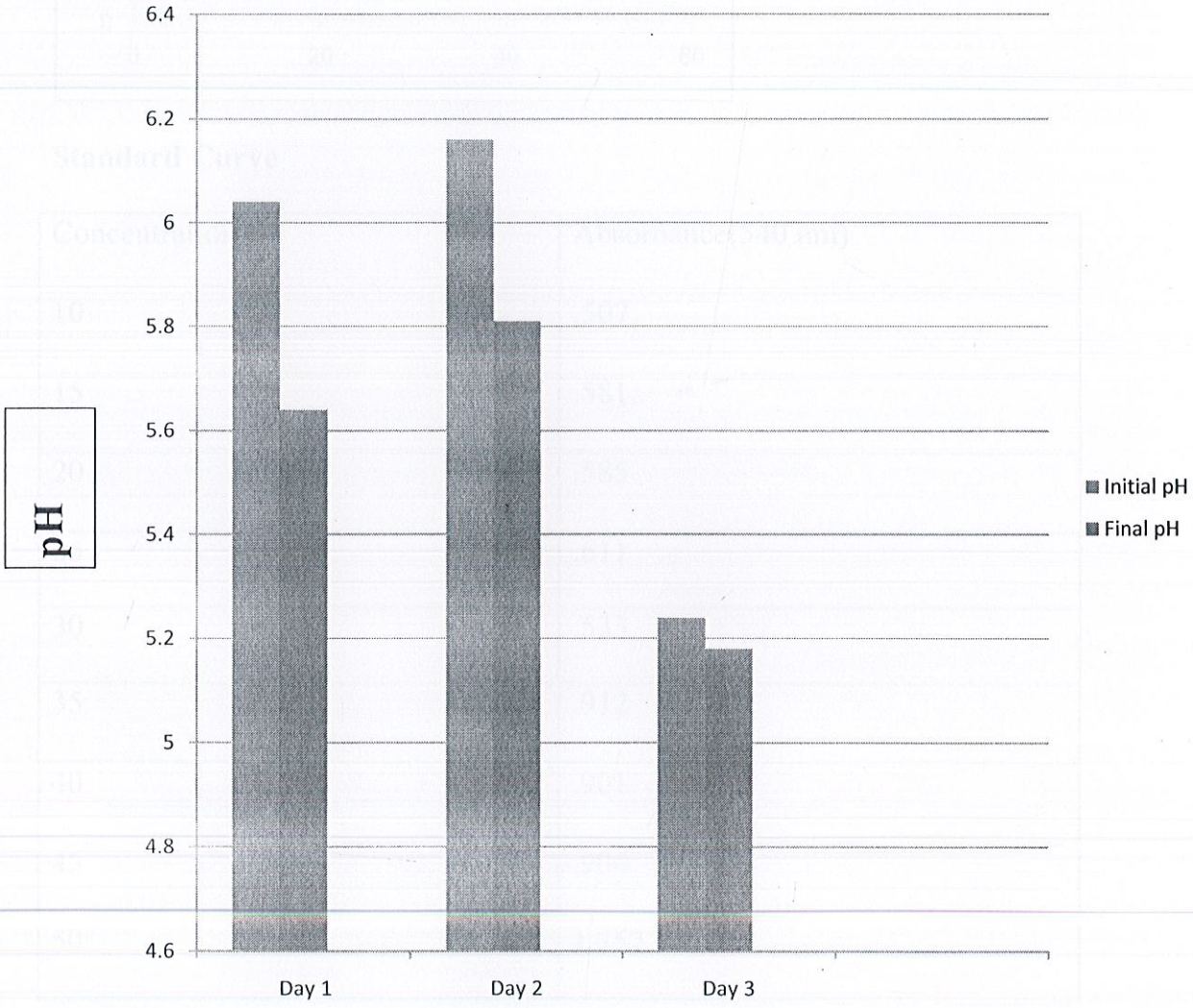
- **The measure the change of pH in water after bubbling CO_2: 28°C, 35 % molasses, Ammonium sulfate 0.1 g/100 ml, 48 hr culture**

Day	Initial pH	Final pH
1	7.12	6.84
2	6.71	6.65
3	6.83	6.72

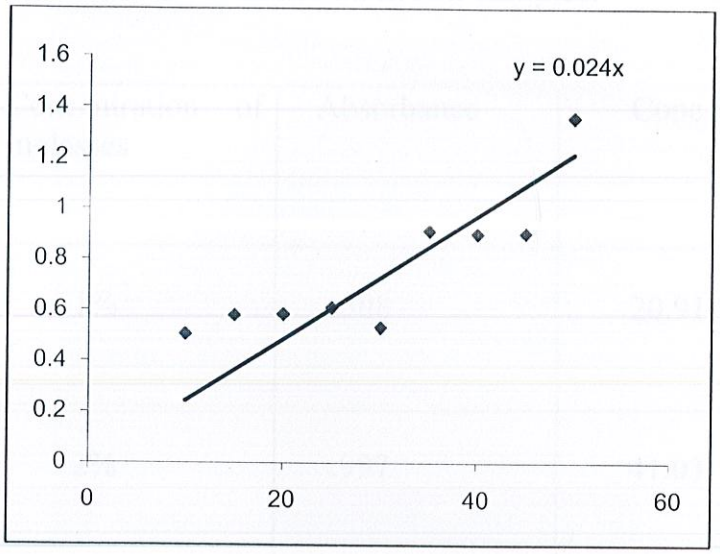


- **The measure the change of pH in water after bubbling CO_2 (24 hr feed): 28°C, 35 % molasses, Ammonium sulfate 0.1 g/100 ml,**

Day	Initial pH	Final pH
1	6.04	5.64
2	6.16	5.81
3	5.24	5.18



- **Reducing Sugar estimation of 35% molasses(Absorbance vs. Concentration)**



Standard Curve

Concentration	Absorbance(540 nm)
10	.507
15	.581
20	.585
25	.611
30	.533
35	.912
40	.901
45	.904
50	1.357

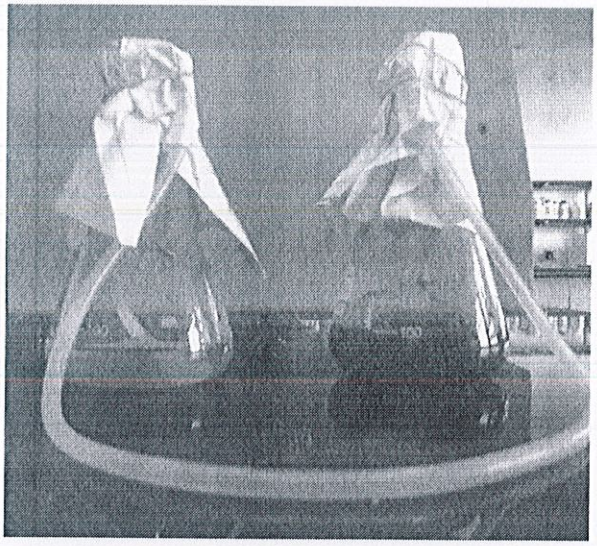
Concentrations of the unknown samples:

Concentration of molasses	Absorbance	Conc %	g/ml
1%	.508	20.91	.2091
2%	.997	41.03	.4103
10%	2.436	100.3	1.002

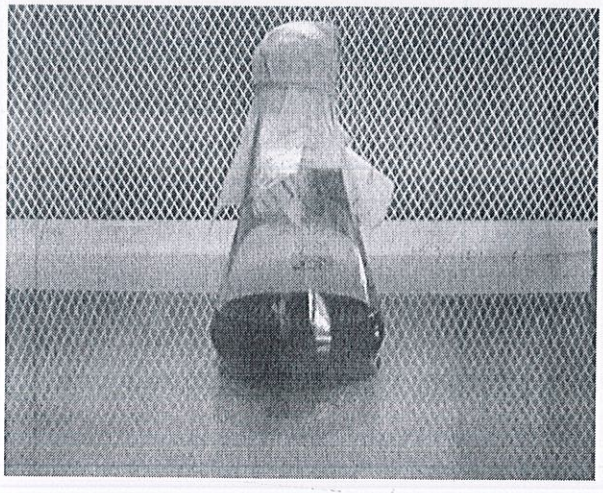
- **Estimation of Spirulina and yeast biomass in coupled system(8 days): Zarrouk's media 50 ml,10 ml Spirulina inoculums,28°Cand yeast 28°C, 35 % molasses, Ammonium sulfate 0.1 g/100 ml, 48 hr culture.**

Spirulina uncoupled: 0.12 gm

Spirulina coupled: 0.34 gm

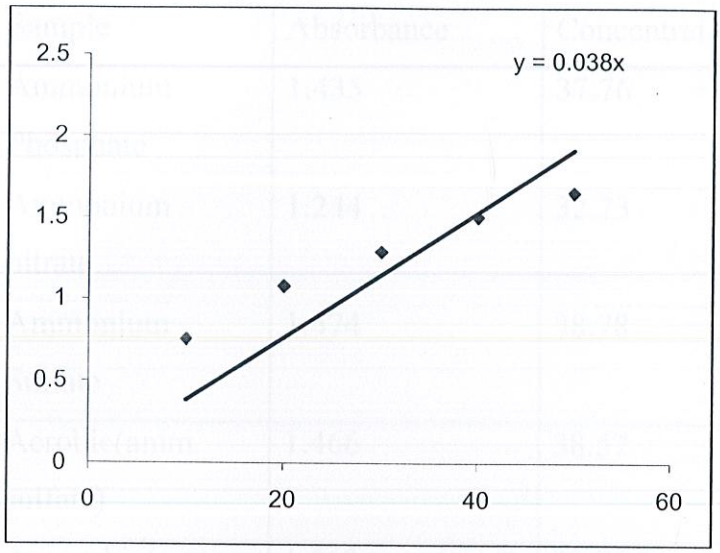


Yeast (ammonium sulfate) biomass coupled: 4.57 gm



- **Protein Estimation(Absorbance vs. Concentration):**

The protein content of the biomass of various nitrogen sources, anaerobic and aerobic system was estimated.



Standard Curve:

Concentration	Absorbance(660 nm)
10	0.756
20	1.083
30	1.293
40	1.505
50	1.655

Concentrations of Unknown samples:

Sample	Absorbance	Concentration %	g/ml
Ammonium Phosphate	1.435	37.76	0.37
Ammonium nitrate	1.244	32.73	0.33
Ammonium Sulfate	1.474	38.78	0.39
Aerobic(amm. sulfate)	1.466	38.57	0.38
Anaerobic(amm. sulfate)	1.663	43.76	0.43

Summary of the results

- After experimenting with three N_2 sources best results were obtained with Ammonium Sulfate.
- The best yield varied between 35-45ml molasses because of the fact that higher sugar concentrations lead to inhibition of growth and promote fermentation.
- The yield was observed at 120 rpm at 4.84 gms.
- The anaerobic conditions over 8 days gave a similar yield. With day2 yield as the highest. This declined over the subsequent days because of growth kinetics of the culture.

- When the yeast culture was unified with a flask of water 2 types of fed-batch processes were carried out, one with a refilling at 48 hrs and the second after 24 hrs but the observation that the maximum pH change in water was at the end of the first day itself.
- The concentration of reducing sugar in 35% molasses was about 50%.
- The Spirulina culture that was coupled with molasses showed more growth than the one that was uncoupled.
- The protein estimation of the yeast samples from different conditions showed a protein percentage between 35-45 %.

Discussions

1. After experimenting with 3 inorganic nitrogen sources we came to the result that ammonium sulfate comparatively gave the best yield while being in the same conditions as other nitrogen sources this could be because of the fact that yeast take up ammonium sulfate better than other inorganic nitrogen sources or the fact that ammonium sulfate gets assimilated much more easier than the other nitrogen sources, the fact being that though the other nitrogen sources gave a comparable results this was the best yield, this result has further implications on the other experiments we have to perform. All other experiments pertaining to different parameters of growth conditions will depend on this initial result for e.g. Rpm, anaerobic and partially anaerobic systems. Hence this result is of importance to all subsequent experiments.

2. The overall result was that the best yield varied between 35-45 ml of molasses for every single nitrogen source this is because of the fact that at lower concentrations the sugars are not able to support the growth potential of the yeast and the carbon source is used up before it completes the exponential phase of growth. The higher concentrations of the sugars inhibit the growth of the yeast because of the fact that increasing sugar concentration create a negative osmotic potential and drain the water from the yeast which is necessary for growth functions hence when yeast starts to lose water it starts to ferment the sugars into alcohol and thus the biomass production is stopped. Hence the observation is that the range of 35-45 is the optimum growth range.

3. The experiment with different rpm showed that the result for 80 rpm indicates the fact that at lower rpm the yield decreases because of the fact that the agitation is lowered and thus the rate of oxygen dissolving into the media is also decreased leading to a situation where oxygen becomes a limiting factor to the biomass production and the system leans towards an anaerobic condition and at a higher rpm the yeast in a state of excess agitation is not able to use up the sugars in the molasses and at higher rpm's the yield decreases, the optimum rpm was found to be 120 rpm.

4. The experiment of the completely anaerobic system gave the result of the maximum yield after 2 days with maximum carbon dioxide evolution at that point of the process with subsequent days the evolution of carbon dioxide decreases to nil and also the yield of the biomass also goes down this is due to the colony growth kinetics that is after the exponential phase the death phase occurs and the biomass starts to die because of reduction of the availability of the sugars and accumulation of the toxic substances. Hence the gradual decline of the biomass.

5. The coupled system with water has a result very typical to the behavior of the yeast in this media. The maximum pH change was observed at the end of the first day and steadily declined at the end; this is consistent with the anaerobic system experiment where the maximum evolution of Carbon dioxide was seen at the end of the first day and the end of the second day.

6. The concentration of reducing sugars in 35%molasses is around 50% which is concurrent with values given in references.

7. The Spirulina was found to have more biomass in the coupled system because of the fact that the carbon dioxide from the molasses was being

redirected to the flask with Spirulina and it is known that Spirulina utilizes the carbon dioxide in the atmosphere photosyntheticaly to grow, thus the biomass in the coupled flask was higher than the solitary flask which had no external augmenting source of Carbon dioxide.

8. The protein estimation of the yeast at various fermentation conditions showed that these have almost the same profile of total protein content the concentration of protein was between 35-45 %, which is about the same as the protein content of yeast. The lack of protein could be explained by the fact that the yeast was grown on molasses which does not give all the amino acids.

Conclusion

The growth of yeast and Spirulina together is a novel idea that uses the natural byproduct of one fermentation, as the substrate to enhance another growth process; hence it is a self dependent process which gives two neutraceutically very important SCP's Yeast and Spirulina. They also have a complementary nutrient profile which is almost complete in its array and entirety.

The anaerobic growth of yeast is strong but the evolution of carbon dioxide is lost after sometime so this has to be worked upon one way is to have a partially anaerobic system.

Ammonium sulfate was the best nitrogen source for the process of yeast biomass growth as it is the inorganic nitrogen source yeast takes up the best. Spirulina is a slow growing alga so the combined process needs to be continued for at least 8 days and to compensate for the reducing sugar loss the molasses is kept in a fed batch process.

The protein content of the yeast at various fermentation conditions was comparable to traditionally grown yeast with a very little deficit in the quantity of protein.

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