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Research Article

Biochemical studies on growth of single cell proteins with yeast extract supplement under varied biotic and abiotic factors

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Abstract

The biological synthesis of SCP based on yeast biomass *Saccharomyces cerevisiae* has attracted considerable attention due to their widespread application as a food supplement. Wastewater obtained from the cassava processing industry was used to produce the single cell protein. Microbial fermentation method was mainly used for the production of single cell protein in the current experiment. The yeast biomass *Saccharomyces cerevisiae* were isolated from the soil sample by serial dilution and the subculture was done by yeast potato dextrose media. The inoculums were maintained by using MYGP media. Wastewater of cassava and the nutrients of cassava were mixed together for production of media preparation. The different variable parameters such as carbon source, nitrogen source, interaction time, temperature and ph were applied to obtain maximum yield of SCP. The crude protein was determined by Lowry's method and growth of the yeast was measured by dried cell weight technique.

Keywords: *Saccharomyces cerevisiae,* cassava wastewater, MYGP media, microbial fermentation, India.

Introduction

Proteins are the macromolecules made up of amino acids which are highly essential for the existence of the living system [1]. The single cell protein which is used as the protein source in animal feeds and protein rich food for humans. The mixed protein derived from the unicellular microbial biomass grown on the biological waste has been used for the production of single cell protein in industry [2]. Many raw materials used for the production of single cell protein have been considered as the carbon and other energy sources. The high protein content present in the single cell protein makes its use attractive, particularly for aquaculture [3].

The role of single cell protein lies partially in the need for more protein and partially as a commercial interest. It acts as the substitution for the conventional protein supplements used mainly in livestock feed [4, 5]. The production of single cell protein assumes special significance on account of its nutritional and physiological function [6]. The waste biomass which is mainly used for the production of single cell protein industrially includes cassava wastes and other agricultural wastes due to the presence of carbon, nitrogen sources for the substantial growth of the microbial biomass. There has been industrial work on the acid hydrolysis of shrimp-shell waste to directly produce protein and indirectly produce single cell protein through a bioconversion process [7]. The main method used industrially for the production of the single cell protein has been microbial fermentation in which microorganisms having high protein content are used [8]. Microorganisms like algae, yeast and bacteria give intensive growth, with optimum food supply at favourable conditions because bacteria are able to double 0.5-2hr, yeast in 1-3hr and algae within 2-6hr [9].

The genetics and chemical composition of these microorganisms can be altered with relative ease because of rapid succession of generation. Single cell protein can be produced from cheap waste raw materials available in large quantities which minimizes environmental pollution [10]. More recently there has been research on the production of single cell protein from carbohydrate sources [11] and liquid waste of plant origin [12, 13].

The various groups of microorganisms that have been considered for food or feed use are algae, bacteria, yeast and moulds [14, 15]. The dried cells of these organisms are collectively referred to as "single cell protein". The term was coined by C.L Wilson of MIT in 1966.

Single cell protein can be produced using a number of different substrates. Often this is done to reduce biological oxygen demand of the effluent streams leaving various types of agricultural processing plants. The various substrates which can be used as the common material for the production of various types of single cell protein includes whey [16], orange peel residue, sweet orange residue, sugarcane bagasse, paper mill waste [17], rice husk, wheat straw residue, cassava waste, sugar beet pulp, coconut waste, grape waste, mango waste, etc. [18, 19]. Different types of algae like chlorella, scenedesmus, and spirulina are commonly used for the production of single cell protein. They use carbon dioxide and sunlight as substrate. Algal SCP has about 60% crude protein which is generally good in amino acid composition except for deficiency in sulphur containing amino acids.

The thermo tolerant filamentous fungi have been used to produce SCP mainly from polysaccharide hydrolysates e.g., [3, 20] starch hydrolysates sulphite liquor from wood pulp industries etc. [21, 22, 23]. They have crude protein content of 50-55%. Yeasts like *Saccharomyces*, *Candida* and *Torulopsis* have been widely studied for SCP production [24]. The SCP has 55-60% crude protein, which has good amino acid balance except for a deficiency in sulphur containing amino acids [7, 25].

A large number of bacterial species have been evaluated for SCP production e.g., *Methylophilus* using methanol SCP has very high crude protein over 80% and good amino acid composition. The economical feasibility of SCP will be dictated by possible uses in the competition with comparable existing products. SCP is rich in protein and can be stored and shipped over a long distance. SCP do not create imbalance in natural ecosystems. No novel synthetic compound is produced and the technology, being based on recycling, is pollution free. In developing SCP processes new technical solutions for other related technologies have been discovered, e.g. in wastewater treatment, production of alcohol and other metabolites, enzyme technology and nutritional sciences. [13, 26, 27].

Materials and Methods

Isolation of the yeast

Yeast is the heterogeneous group of fungi that superficially appear to be homogenious. The majority of ascomycetous and basidomycetous yeasts isolated in the laboratory go unrecognized because most of them are heterothallic. Yeast like fungi reproduce asexually. The identification of these fungi is based upon a combination of morphological and biochemical criteria. Morphology is primarily used to establish the genera, whereas biochemical assimilations are used to differentiate the various species.

Isolation technique for mixed cultures

All the initially isolated yeasts of the current experiment were contaminated in the mixed culture. The purification of isolated yeast was done by direct mounts and subsequent streaking through colony isolation. Pure cultures were used for assimilation procedure of the current experiment. Spatial isolation of streaking subcultures was considered for the purification of the yeast. The additional techniques used for the purification of yeast are listed below.

Serial dilution method

The serial dilution of the microorganism under experimentation was done by serial dilution method in liquid medium. The different dilutions considered for the current experiment were 1ml of the medium to 9ml of the sterile water for 1:10 dilution, 1ml of the 1:10 dilution to 9ml of the water for 1:100 dilutions and so on. The number of yeast per millilitre of the fluid was reduced by 9/10 in each dilution. Subsequent dilutions were made in ratios of 1:1000, 1:10, 000, 1:1, 00, 000, 1:1,000,000 or even 1:1:10,000,000 the original culture contained an extremely large number of organisms.

From each dilution of the current experiment usually with 1:100, 1ml of the culture was transferred to a sterile agar plate. Spread plate methodology was used for the transfer of the microorganism under experimentation. All the experiments were done under purely aseptic conditions. In the spread plate technique the sample was first placed on the centre of the solid cooled agar medium in the sterile petriplate. The sample was then spread evenly over the medium surface with sterile, blend glass rod. The colonies developed on the surface of the agar were observed after incubation.

Principle criteria and tests for identifying yeast culture characteristics

Direct mounts

Direct mounts were made in order to study yeast morphology microscopically and to determine purity of the isolates.

Lacto phenol blue staining

The fungal suspension was prepared with lacto phenol cotton blue stain. A small drop of lacto phenol was placed on a clean glass microscope slide and a small portion of the yeast colony formed was removed and placed into a drop of lacto phenol. A clean cover glass was placed over the suspended cells and observed microscopically. The suspension was covered with the cover slip and the cover glass was sealed with fingernail polish to temporarily preserve the mount. The whole setup was viewed under microscope indicated in Figure 1.

Sub culturing

Initially YPD media was prepared aseptically and filled in two test-tubes each having known volume in ml of the YPD media and the slants were made by allowing the medium to solidify after a particular

interval of time. Then the isolated sample was inoculated in the solidified slant and kept in a test tube with solidified sub cultured media inside the incubator at room temperature for one day.

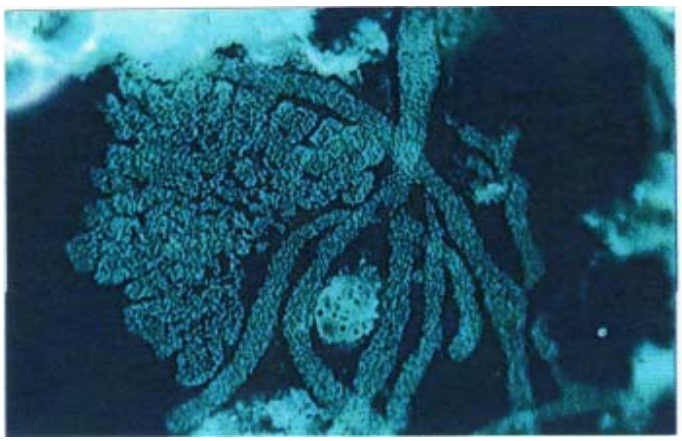

Figure 1. Microscopic View of Yeast Cultured after Lacto Phenol Test.

Inoculum maintenance

The inoculum maintenance was done by keeping the test tubes inside the fridge in MYGP media at 4°C for future use.

Optimization of culture media

Different media such as sucrose, glucose and cassava were optimized for different variable parameters such as pH, temperature, carbon source and nitrogen source. Finally cassava media was found to be the media with maximum growth.

pH

For studying the amount of protein content in the broth culture of cassava nutrients and wastewater of cassava was optimized at pH 6, 7, 8.

Temperature

The cassava processing wastewater and cassava were mixed well and after inoculation they were kept at room temperature and also in the rotary shake incubator at 37°C.

Carbon source

To determine the best biomass yield in different carbon sources like sucrose, glucose and maltose, these were dissolved separately in cassava nutrient media and maintained at pH 6 then sterilized and placed in the rotary shaker incubator for 48 hrs.

Nitrogen source

Except for yeast extract and urea, all other chemicals of cassava nutrient medium were dissolved separately in peptone, sodium nitrate, beef extract and ammonium sulphate, maintaining pH at 6 and then after inoculation of *Saccharomyces cerevisiae* all the flasks were placed in rotary shaker incubator for 48hrs.

Production media preparation
Wastewater obtained from cassava processing industry was sterilized and then the nutrient for the cassava media was taken and sterilized. After sterilization both cassava wastewater and nutrient for cassava media were cooled and mixed properly.

Inoculation
In this process cassava ingredients were prepared and dissolved in cassava wastewater. Then both media were inoculated inside the laminar air flow using the culture slants previously kept in the freezer at 4°C .All the flasks were kept in the rotary shaker incubator at 37°C or room temperature for the growth of the yeast.

Mass Production
Inoculum preparation for fermentation
20 ml of YPD media was taken and maintained at pH 6 before autoclaving. Then inoculation was done in broth media and placed in room temperature.

Bulk media preparation
One litre of wastewater obtained from the cassava processing industry was taken and sterilized inside the fermentation after autoclaving and cooled for 30 minutes.

Fermentation
A batch fermenter was used for the production of SCP from wastewater of cassava. The experiment set consisted of the fermenter, the air supply and the computer based data acquisition control system. The fermenter and all accessories were chemically sterilized 2% potassium meta bisulphate solution and then washed with hot water for several times. The reactor was then filled with one litre of cassava wastewater and immediately inoculated with 20ml of the inoculum. Cell suspensions were prepared from 24hr old cultures in sterile media and aseptically to the one litre flask containing cassava wastewater and pH of the medium was adjusted at 6 before autoclaving. The pH was maintained at 6 with the addition of HCL and NaOH using computer based pH measurement and control system. Continuously monitored dissolved oxygen and temperature and the reactor was operated at 2vvp at mixing speed of 300rpm. The samples were taken after 48hrs for maximum yield of biomass.

Isolation of single cell protein
10 ml of the solution was fermented and after that centrifuged at 6000rpm for 20 minutes. Then the supernatant was collected.

Purification methods
The supernatant obtained containing enzyme lipase was subjected to purification. Main steps for purification of the current experimentation are listed below.

Ammonium sulphate precipitation or salting out
Ammonium sulphate of 40% w/v was added to the cell free supernatant and was stirred for 4 hrs. The procedure was done at 4°C. The precipitate obtained was allowed to stand for two hrs and then collected by centrifugation at 15000 rpm for 20 minutes. The pellet obtained was dissolved in 2ml of 2mM glycine NaOH buffer, pH at 11 and protease activity was assayed.

Dialysis

Preparation of dialysis tubing

The dialysis tube of required length was boiled once in distilled water, containing a pinch of sodium and EDTA and then thrice distilled water.

One end of the dialysis tube was tied with thread. The tube was then filled with the protein solution to be purified. The sealed tubing was suspended in about 250ml of 25mM glycine NaOH buffer at pH 11.0. The setup was continuously stirred by means of a magnetic stirrer and the temperature was maintained at 4°C glycine NaOH buffer, pH 11.0 was changed 4 times at an interval of two hrs.

Dialysis was done extensively until the protein concentrate was obtained and its protease activity was assayed. The protein concentrate was then subjected to ion exchange chromatography.

Ion –exchange chromatography

In this method, protein in the solution with a low ionic strength was added to the column. The protein interacted with the beads via electrostatic interaction. In anion exchange chromatography, the matrix carried out a net positive charge which interacts with negatively charged protein strongly. The removal of the protein from the column was achieved by washing the column with a buffer of decreasing pH as the pH lowers, the charge of the protein is reduced, leading to the weaker interaction with positively charged matrix. In cation exchange chromatography the matrix was negatively charged and it interacts with positively charged proteins. Elution was again achieved by a gradient of ionic strength or by an increasing pH gradient. The method relied on having two pumps which can control the mixing of low and high ionic strength to form the buffer gradient that washes the column.Seperation still depended upon "theoretical plate" model because the protein is bound in a sharp band at the start of the column. As it was eluted it begins to pass down the column is still determined by its ability to re-associate with the stationary phase. The separation was achieved by two methods such as binding strength of protein at a given ionic strength and partition constant as it passed down the column after its initial displacement from the start of the column.

Activation of the column

The column material was soaked in distilled water for 1-2hrs in a beaker. The material was then transferred into 50ml of 0.01M NaOH and was allowed to stand for 30 minutes. The above procedure was repeated twice. The matrix material was washed 3-4 times in distilled water for 30 minutes. The material was then transferred to 50ml 0.1N Hcl and was allowed to stand for 30 minutes. The above step was repeated twice. After washing the matrix material-DEAE.Sephadex was transferred to the glass column and was equilibrated with citrate phosphate buffer with pH of 6.2.

Fractionation of dialyzed protein

The dialysis protein was subjected to exchanger, DEAE-Sephadex column. The adsorbed protease was eluted by linear gradient of NaCl from zero to 250mM in the same buffer. Protease activity was assayed in each 1.0 ml of the 4ml h. The number of fractions obtained were 11. In final step, the active fractions were pooled, concentrated by polyethylene glycol and dialyzed against citrate phosphate buffer, pH at 6.2 overnight.

SDS –PAGE

The anionic detergent Sodium Dodecyl Sulphate (SDS) dissociates proteins in their constituent polypeptide chains. Monomeric SDS binds tightly to most of the proteins at about 1.4 mg SDS/mg protein. Combined treatment with disulphide reducing agent completely unfolds the protein.

Poly acryl amide gel electrophoresis in the presence of SDS separates the polypeptide chains according of molecular weight. Thus molecular weight of the polypeptide chains of a given protein can be determined by comparing their electrophoretic mobility on SDS gels to the mobility of marker proteins with polypeptide chains of known molecular weight.

The most popular electrophoresis method is the SDS-PAGE system developed by Laemmli. This is a discontinuous system consisting of two gels: Stacking gel. The samples are prepared in low conductivity buffer (60mM Tris-Cl ,pH=6.8) and are loaded between the higher conductivity electrode buffer (25mM Tris,192mM glycine , pH =8.3) and the stacking gel (125mM Tris-Cl ,pH=6.8). When the power is applied, a moving boundary region is rapidly formed with the highly mobile chloride ions in front and relatively slow glycinate ions in the rear. A localized high voltage gradient forms between the fronts, causing the SDS-protein complex to condense into a very narrow region and migrate between the chloride and glycinate phases. The total amount of the SDS has a considerable influence on the resolution. High concentrations of SDS can lead to broadening and spread the protein bands. At the interface of the stacking and separating gel the SDS-protein complex experience a sharp increase in retardation due to the restrictive pore size of the resolving gel. The glycinate ions overtake the proteins, which then move in the space of uniform pH formed by the Tris and the glycine.

The separating gel

The glass plates, spacers and comb were cleaned with detergent and rinsed with water and dried. The glass plates were wiped with ethanol. The glass plates were sandwiched, depending on the gel system used.

The separating gel solution was prepared, except ammonium per sulphate and TEMED, in a vacuum flask. It was then de-aerated for 1 minute under vacuum. After adding the ammonium per sulphate and TEMED were mixed with the solution gently. With the help of the pipette the separating gel solution was filled between the glass plates sandwiched along the edge of the spacers, until the height of the solution was 1.0cm below the comb. Immediately the solution was overlayed with water saturated 2-utanol or isopropanol to exclude air and to obtain an even interface between the gels. The gels were allowed to polymerize for 45 minutes. The gel was polymerized when sharp interface was viewed below to overlay.

The stacking gel (5ml)

The stacking gel was prepared just before using the gel to maintain the ion discontinuities at the interface between the two gels. The ingredients were mixed and de-aerated before adding the ammonium per sulphate and TEMED.

The solution was removed from the top of the gel, rinsed with water and the area above the gel carefully with the filter paper was dry yield. The stacking gel solution was filled on the top of the separating gel. The well forming comb was placed in the positions, being careful not to trap air bubbles under the teeth. Visible polymerization of the gel should occur within 20 min.

3X sample buffer preparation: 3 ratio sample (Sample:Buffer)

The SDS-reducing sample buffer was prepared by adding 100µl 2-mercaptoethanol to each 0.9ml sample buffer. The sample (10µl) was diluted with an equal volume of SDS-reducing sample buffer. The samples were heated for 3 minutes at 95°C. Hydrophobic (membrane) proteins were incubated at 15 minutes for 37°C.

Assembling
The electrophoresis cell was assembled; tubing, clamps and comb were removed. The upper and lower reservoirs were filled with electrode. The samples were loaded into the wells in the stacking gel. The electrophoresis unit was connected to the power supply. The lower electrode was anode (+) and the upper was cathode (-). Electrophoresis was started with low voltage (70volt) and increased to higher (200volt) until the blue dye had reached the bottom of the gel. The 10X buffer was diluted as per requirement with water.

De-staining
The gel was soaked in excess of staining solution for 1hr. It was de-stained with solution overnight and observed for bands.

Protein estimation
Protein estimation was done to quantify the amount of protein present in the sample by two methods:-

Lowry's method
For protein estimation the culture was transferred into the Eppendorf and centrifuged at 8000rpm for 12 minutes. Then the pellet was taken and dissolved in 1ml of phosphate buffer for protein estimation. It was then analyzed by UV spectroscopy.

Beer-lamberts law
When the beam of monochromatic light passes through the solution containing absorbing material, the intensity of the light decreases as the light of the absorbing medium increases. It was then analyzed by UV spectroscopy.

Procedure
0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 1ml of the working standard were pipetted into the series of test-tubes. 0.1ml and 0.2ml of the sample extracts were pipetted into the two other test-tubes. The volume was made to 1ml in all the test-tubes. A tube with 1ml of the water served as the control. The volume was made up to 5ml by adding the solution containing florescent particles. The OD was measured at 280nm.

Lowry's method
The blue colour developed by the phosphomolybdic phosphor in the folin ciocalteau by the amino acid tyrosine and the tryptophan present in the protein plus the colour developed by the biuretic reaction of the protein with the alkaline cupric tartarate are measured by Lowry's method [1].

Procedure
The procedure was conducted as described above. Then 5ml of the reagent C was added to each test-tube including the blank. It was mixed well and allowed to stand for 10 minutes. The 5ml of the reagent D was added to each tube including the blank. It was mixed well and incubated at room temperature in the dark for 30 minutes. Blue colour developed. The reading was taken at 660 nm in the colorimeter.

Results and Discussion

Yeast microorganisms were extracted and identification of the yeast was done by gram staining, Lacto-phenol test. Optimization of media was done for pH, temperature, carbon source and nitrogen source and cassava media was selected as the production media. The yeast inoculated in cassava media was transferred to the fermenter. After two days SCP was extracted by centrifugation. The purification was carried out by salting out method, dialysis, ion exchange chromatography and finally by SDS page. Protein estimation was done by spectroscopy using Lowry's method. Following are the results obtained.

Gram staining

The strain that appeared as purple colour, round shaped and showing budding is *Saccharomyces cerevisiae.*

Lacto phenol test

The cytoplasm was seen as a lightly stained blue region, forming a round shape and budding. Microscopic view of yeast cultured after lacto phenol test was shown in Figure 1.

pH optimization

The optimum pH was found to be 6, as shown in Figure 2 and the OD values obtained at different pH are shown in Table 1.

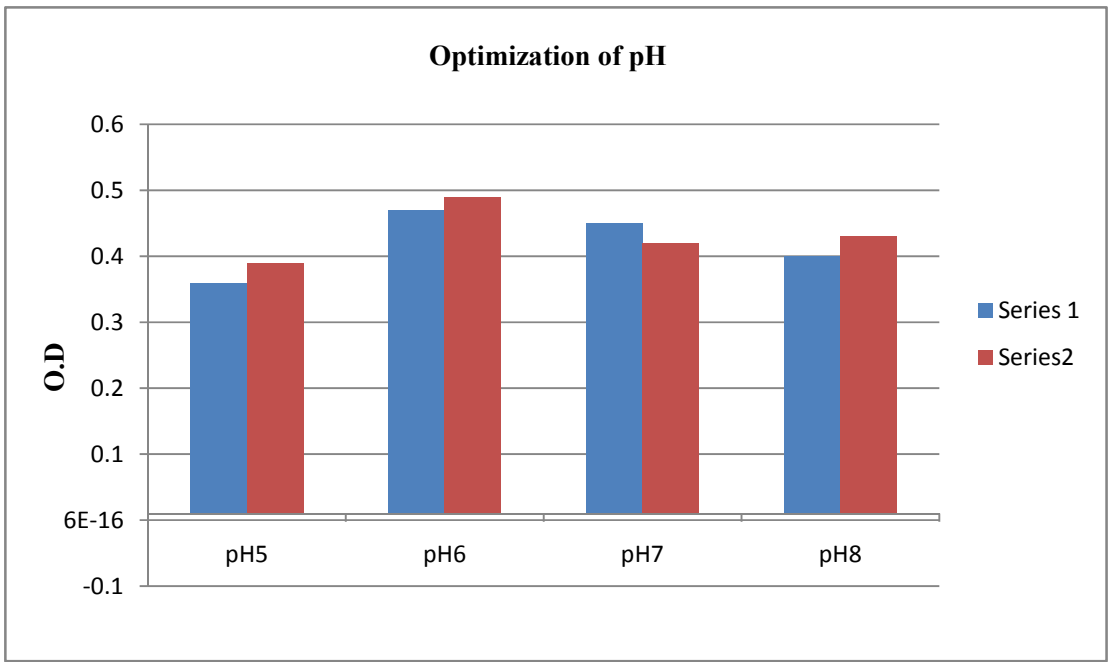

Figure 2. Graph showing pH optimization.

Table 1. OD values obtained at different pH.

pH	Concentration	OD
5	0.1	0.35
	0.2	0.39
6*	0.1	0.46
	0.2	0.49
7	0.1	0.44
	0.2	0.41
8	0.1	0.41
	0.2	0.42

Temperature optimization

The optimum temperature was found to be 28°C, as shown in Figure 3 and the different and the OD values obtained at different temperature are shown in Table 2.

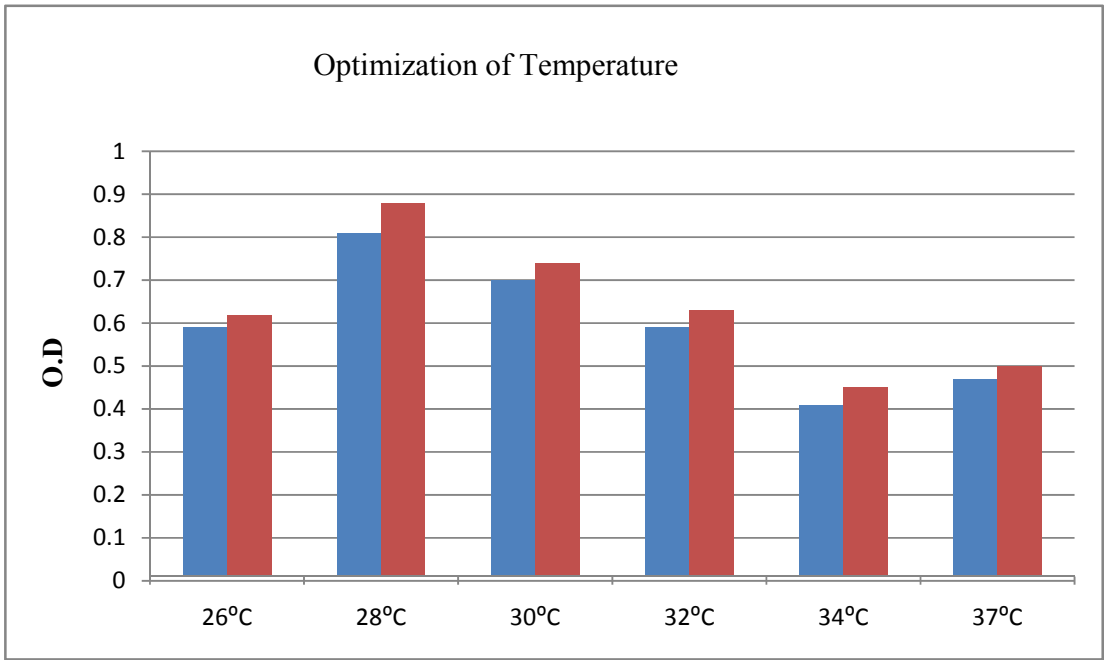

Figure 3. Graph showing temperature optimization.

Table 2. OD values obtained at different temperatures.

Temperature	Concentration	OD
26	0.1	0.59
	0.2	0.63
28*	0.1	0.81
	0.2	0.87
30	0.1	0.70
	0.2	0.74
32	0.1	0.58
	0.2	0.62
34	0.1	0.41

Optimization of carbon source

It can be seen from Figure 4 that cassava media was found to have maximum carbon content and different OD values obtained for different carbon sources are shown in Table 3.

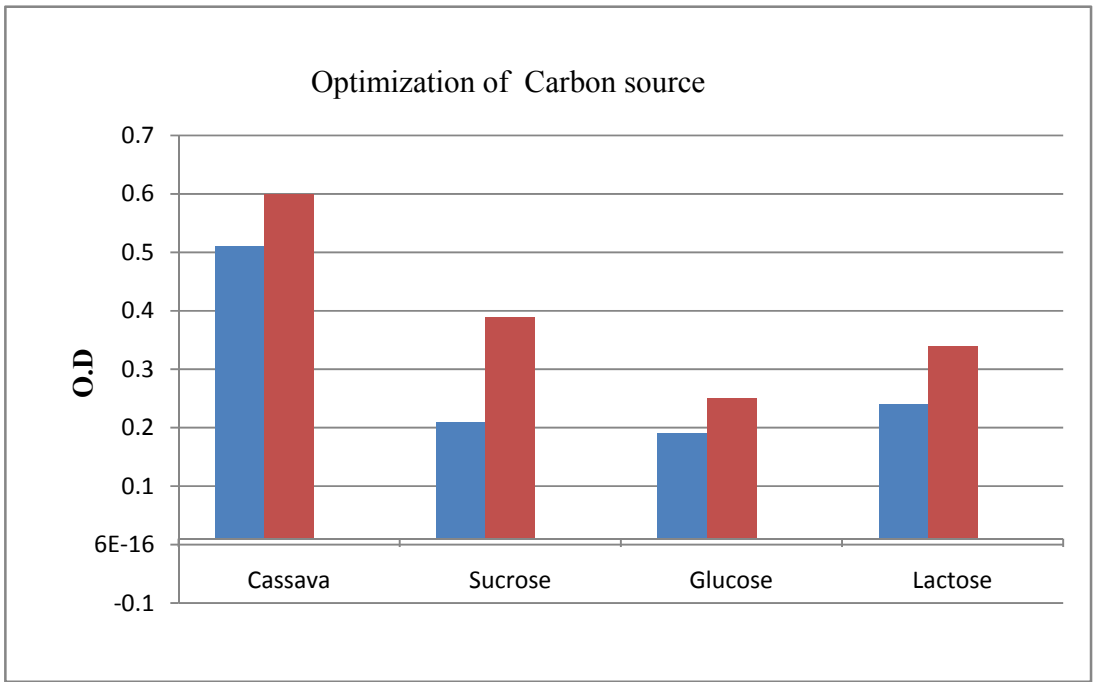

Figure 4. Graph showing carbon source optimization.

Table 3. OD values for different carbon sources.

Carbon Source	Concentration	OD
Cassava*	0.1	0.51
	0.2	0.60
Sucrose	0.1	0.21
	0.2	0.38
Glucose	0.1	0.19
	0.2	0.26
Lactose	0.1	0.23
	0.2	0.35

Optimization of nitrogen source

It can be seem from Figure 5 that the cassava media had the maximum nitrogen content and different OD values obtained for different media are shown in Table 4.

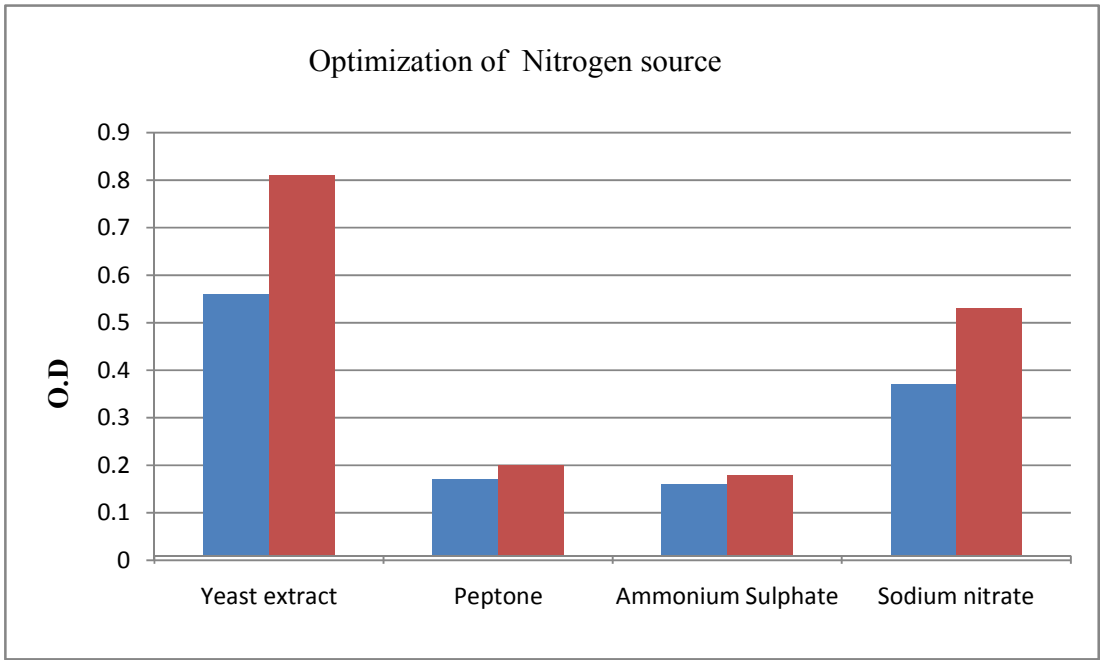

Figure 5. Graph showing nitrogen source optimization.

Table 4. OD values for different nitrogen sources.

Media	Concentration	OD
Yeast Extract*	0.1	0.56
	0.2	0.81
Peptone	0.1	0.18
	0.2	0.20
Ammonium Sulphate	0.1	0.16
	0.2	0.18
Sodium nitrate	0.1	0.36
	0.2	0.52

Protein estimation

Figure 6 graphically shows UV spectrophotometric analysis by Beer Lamberts Law for protein samples at different concentrations. The samples having 5mMNaOH shows maximum OD value at 280nm and the OD values obtained from different samples at 280nm are shown in Table 5.

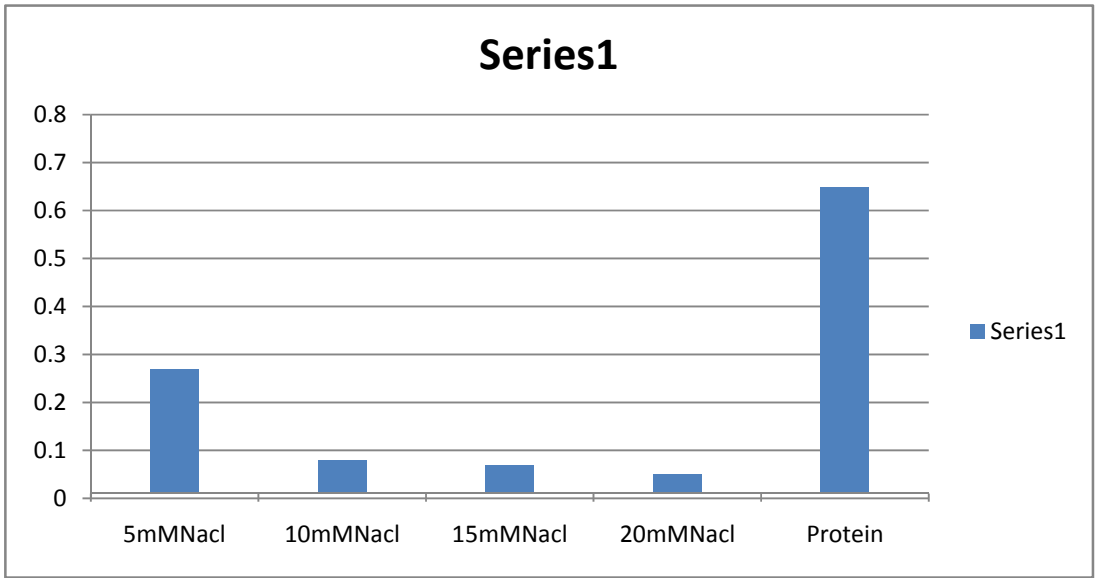

Figure 6. Graph showing protein samples at different concentrations by Beer Lambert's Law.

Table 5. Readings obtained at different concentrations using Beer Lambert's Law.

S. No.	Samples	OD at 280nm
1	5mM Nacl	0.276
2	10mM Nacl	0.073
3	15mM Nacl	0.051
4	20mM Nacl	0.034
5	Protein	0.633

The graph in Figure 7 shows UV spectrophotometric analysis by Lowry's method for protein samples at different concentrations. The samples having 5mMNaOH show maximum OD value at 280nm and the OD values obtained from different samples at 280nm are shown in Table 6.

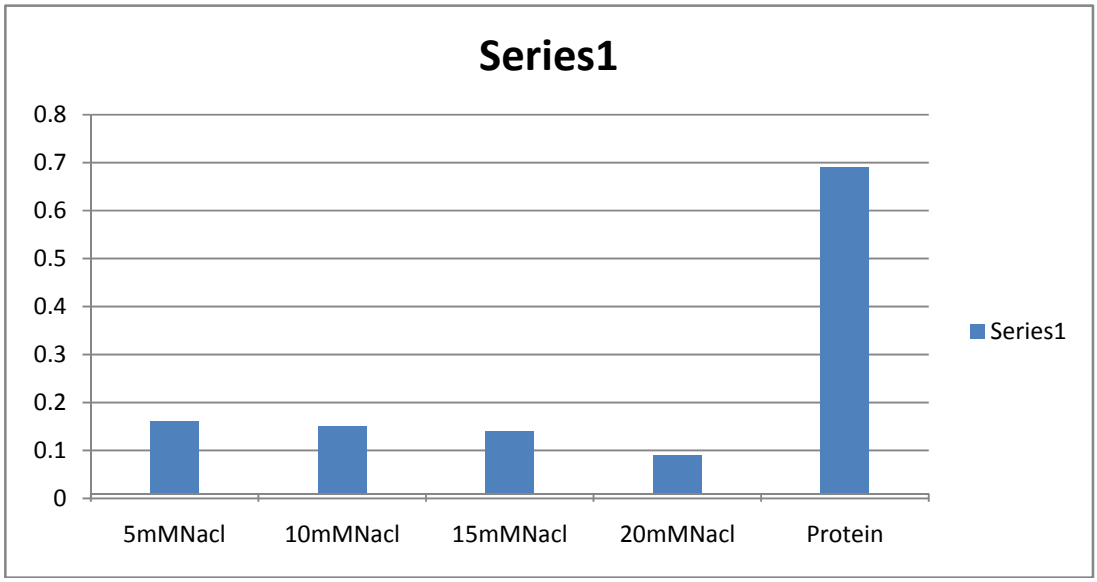

Figure 7. Graph showing protein samples at different concentration by Lowry's Method.

Table 6. Readings obtained at different concentrations by using Lowry's Method.

S. No.	Samples	OD at 280nm
1	5mM Nacl	0.17
2	10mM Nacl	0.16
3	15mM Nacl	0.15
4	20mM Nacl	0.091
5	Protein	0.691

Ammonium sulphate precipitation or salting out

The precipitate was allowed to stand for two hours and then pellets were collected after centrifugation at 15000gm for 20 minutes.

Dialysis

Dialysis was done and small molecular weight molecules were removed.

Figure 8. SDS-PAGE Electrophoresis.

Ion exchange chromatography
Proteins were separated by ion exchange chromatography.

SDS-PAGE
The different protein concentrations obtained were identified by SDS-PAGE run through electrophoresis and are shown by the dark bands in Figure 8. The molecular weight of the suspected SCP is 66KDa. The current experiment obtained ends at 60 and 52 KDa showing the SCP.

Conclusion

Cassava processing wastewater was found to be acidic. *Saccharomyces* species have indicated that limitation of nitrogen sources. The addition of different nitrogen sources exerted no noticeable effects on the yeast yield. No correlation was shown between cell mass yield and efficiency of the cells in producing proteins. Thus the medium containing yeast extracts supported best growth. The pH of the medium was maintained at 5, 6, 7 and 8 during the course of experiments. The optimum growth of *S. cerevisiae* was achieved at pH6. The SCP production of yeast was affected by temperature. The various temperature conditions were tested but at room temperature it gives highest yield. As SCP productions were intracellular proteins it requires high speed agitation during the fermentation for maximal propagation of yeast. The dry yeast was taken for its content of crude protein. SCP from wastewater materials minimizes the environmental pollution. These can be used as protein sources in human food or animal feed. In the present study, the media was optimized for production of SCP. The optimum parameters obtained were:

pH - 6
Temperature - 28°C
Carbon source - 0.6(cassava media)
Nitrogen Source - 0.82(yeast source)

Thus the protein content was quantified in the SCP. The protein purified using salting out, dialysis, ion exchange chromatography methods and its high content was confirmed. SDS page undertaken and the protein bands were obtained at 60 and 52 KDa showing SCPs.

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