

# SINGLE CELL PROTEIN PRODUCTION WITH *LACTOBACILLUS* SPECIES AND *BACILLUS CLAUSII* ON MEDIA CONTAINING SUGARCANE BAGASSE EXTRACT & FISH WASTE HYDROLYSATE

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**Abstract:** Single cell proteins are dried cells of microorganisms used as protein source in human and animal feed. Microorganisms such as algae, bacteria, fungi utilize cheap feedstock/waste for growth and develop biomass and protein concentrates intra cellularly. Alarming increasing rates of population and increased pressure on agriculture base food production urges us to search for alternative protein sources. Bacteria such as *Lactobacillus* species and *Bacillus clausii* are edible, safe and probiotic bacteria in which SCP production can be ventured. In this project Sugarcane bagasse and Fish waste were employed as carbohydrate and nitrogen source respectively.

Sugarcane bagasse is a cheap and abundant lignocellulosic agricultural waste. It mainly contains cellulose (polysaccharide) which can be converted into monosaccharides like glucose through chemical treatment to be utilised as fermentable carbohydrate source. Fish waste includes skin, head, viscera, trimmings, liver, bones etc. These by products contain a good amount of protein rich material which can be converted to fish protein hydrolysates (FPH). This hydrolysate acts as great nitrogen source A submerged fermentation of these bacterial species at room temperature with aeration gave sufficient product over the span of 7 days. Both the bacteria produced higher amounts of intracellular protein when grown on media formulated with sugarcane bagasse extract and fish waste hydrolysate compared to the synthetic growth media. Hence agricultural and fish industry waste can be utilised into value added product like SCP.

**Keywords:** Single cell protein, sugarcane bagasse, fish viscera, fermentation, *Bacillus clausii*, *Lactobacillus*.

## I. INTRODUCTION

Rapidly increasing world population has posed the challenge of providing alternative sources of nutrition to the humankind. In particular protein supplies pose a problem since essential amino acids cannot be replaced. India, a developing country is facing nutrition deficiency and food scarcity problems (Gour *et al*, 2015). Kwashiorkor a type of protein energy malnutrition was observed to be cause of 69% of death in children below the age of 5 in India (2019). In the face of such issues single cell proteins derived from waste organic products could prove to be a useful technology.

The term SCP was coined in 1966 by Carol L. Wilson. Single cell proteins are the dried microbial cells which are used as protein supplements (A.T. Nasser *et al*, 2011). Besides high protein content (60-80% of dry cell weight), SCP also contains fats, carbohydrates, nucleic acids, vitamins and minerals. Another mentionable advantage of SCP is that it contains two essential amino acids, namely lysine and methionine which are limiting in most plant and animal originating food (Gour *et al*, 2015).

Production of protein from microbial source has high significance as it is an alternative source of high-quality supplementation of staple diet. Large scale processes for SCP production show interesting features such as:

- A vast range of microorganisms, substrates and methodologies can be used.
- The fast growth rate of microorganisms provides high productivity.
- Independence of seasonal or climatic factors.
- Does not require large expanse of land. (A.T. Nasser *et al*, 2011; K. Spalvins *et al*, 2018)

SCP can also be derived from bioconversion of agricultural and industrial waste. Use of such waste can results in lowered cost of final product. The biodegradable agricultural waste can be used to produce value added products such as single cell oil (Finco *et al*, 2016), building block chemicals (Wery & Peterson, 2004; Fitzpatrick *et al*, 2010) and SCP. Out of all types of agricultural waste lignocellulosic materials stand out as cheap, abundant and renewable substrates. The biomass is mainly composed of cellulose (30-56%), lignin (3-30%), hemicellulose (10-24%) and proteins (3-7.2%). After undergoing pre-treatment and hydrolysis the polysaccharides can be converted to fermentable sugars (K. Spalvins *et al*, 2018).

India is second largest producer of sugarcane right after Brazil and hence a large amount of sugarcane bagasse is produced after sugar production. Bagasse is the lignocellulosic waste. India produces about 80 million metric tons of bagasse every year (India:

Bagasse, production (thousand metric tons), www.factfish.com), out of which only a small amount is used in cogeneration plants for electricity production. Hence sugarcane bagasse stands out as a great source of carbohydrates in the SCP production.

Along with sugarcane the other substrate used for this project is fish waste which includes skin, head, waste, trimmings, liver, bones, frames etc. These components can contribute 40-60% of total fish produce. These by products contain a good amount of protein rich material which can be converted to fish protein hydrolysates (FPH). They are mixture of polypeptides, dipeptides and amino acids (Dede and Tati, 2016). As of 2015-16 out of 1.15 million tons about 0.55 million tons that is 50% of fish produce is disposed as waste in India (Waseem Ahmad & Santosh Bhuimbar, 2019).

SCP can be produced by variety of microorganisms such as algae, bacteria, fungi out of which Bacterial cells are more preferred as they have high protein content (50-80%), rapid growth and short generation time (A.T. Nasser *et al.*, 2011). The selected species *Lactobacillus* and *Bacillus clausii* for current research work are suggested to be edible and extensively used in probiotic industry. The species of *Lactobacillus* such as *L. acidophilus* has been used in SCP production with stick water (fish meal factory waste) previously (Safaribi Kam *et al.*, 2012), as well as that *L. pentosus* is observed to grow on sugarcane bagasse hydrolysate (Daiana Wischral *et al.*, 2018). Due to its easy accessibility and versatility to grow on wide range of substrate the *Lactobacillus* species was selected which was isolated from homemade yogurt.

The *Bacillus* species especially *B. licheniformis* and *B. subtilis* have been used for SCP production with Tuna fish waste (Safari R. & Yaghoobzadeh Z., 2012). The *Bacillus subtilis* species holds the potential to produce SCP on cellulose, hemicellulose as well as non-protein nitrogenous compounds. *Bacillus clausii*, an acid and heat stable spore former is currently the probiotic, mainly used for diarrhoea treatment (Jayanthi N. & Ratna Sudha M., 2015). Due to its pre-existing probiotic properties and scarce use in SCP production, it stands out as the novel candidate for SCP production. A commercially available spore suspension of *Bacillus clausii* is employed for the process.

The production of SCP takes place through a fermentation process. The selected strains of microorganisms are multiplied on suitable raw material in technical cultivation process directed to the growth of the culture and cell mass and followed by separation processes. In current research, submerged fermentation technique coupled with aeration was found to be suitable for high cell mass as well as for SCP production.

## II. MATERIALS AND METHODS

### 2.1 Isolation of microorganisms, enrichment, identification, optimisation

*Lactobacillus* species was isolated from a homemade curd sample whereas *Bacillus clausii* was obtained from commercially available spore suspension under the name 'Tufpro'. For enrichment purposes MRS broth for *Lactobacillus* species and Nutrient broth for *Bacillus clausii* were utilised. The enrichment was carried out at 28°C with aeration for 24 hours. Isolation was carried out on same media.

For identification Gram staining as well as biochemical analysis was carried out. Sugar fermentation tests, Catalase test, Oxidase test, Oxidative fermentative test, Casein digestion (*Lactobacillus*) starch hydrolysis and nitrate reduction (*Bacillus clausii*) were performed.

The carbohydrate content optimisation was carried out by minimum inhibitory concentration (MIC) estimation by broth dilution technique for both bacteria. The extract prepared from sugarcane bagasse was used for this purpose.

### 2.2 Substrate selection, hydrolysis, quantitation

Sugarcane bagasse and fish waste from Surmai (*Scomberomorus guttatus*) were selected as carbohydrate and nitrogen source respectively. The sugarcane bagasse used for this project was obtained from a local juice vendor. It was dried at 50°C for 24 hours and then grinded to get powder. The pretreatment of bagasse was done with 1% v/v H<sub>2</sub>SO<sub>4</sub> at 121°C for 15-20 minutes in order to remove lignin. The substrate was washed with distilled water, dried and weighed. The dried bagasse was treated with 1.5% v/v NaOH at 10°C for 1 hour, after 1 hour the bagasse was washed with distilled water, dried and weighed. Bagasse was then acid hydrolyzed with 10% v/v H<sub>2</sub>SO<sub>4</sub> at 121°C for 15-20 minutes, this treated substrate was then filtered and the filtrate was neutralized with NaOH solution.

A locally available fish variety called Surmai (*Scomberomorus guttatus*) selected as the preferred protein (nitrogen) source. The fish is easily available and provides more amount of waste due to its large size. For the conversion of fish waste into nitrogen/protein source, the commercially available enzyme papain was used. The fresh Surmai waste was washed and cleaned and converted into paste with food processor. The paste was stored at -4°C for further use. At the time of use the paste was thawed and homogenized with distilled water in the ratio 1:2. The pH of the homogenate was set to neutral. 0.26% of papain solution was added to the homogenate and hydrolysis at 60°C for 3 hours was carried out followed by enzyme inactivation at 85°C for 15 minutes. The obtained hydrolysate was centrifuged at 8000 RPM for 15 minutes and the middle liquid fraction was separated. This protein containing fraction was stored at 5°C for further use.

The total reducing sugars present in bagasse extract were quantitated by DNSA technique. The total protein estimation in fish waste hydrolysate was done by Folin-Lowry technique.

### 2.3 Fermentation for SCP production

Submerged batch fermentation was conducted at 28°C with aeration for 1 week. For control, standard synthetic media, MRS broth for *Lactobacillus* and Nutrient broth for *Bacillus clausii* were used. Effect of only C & N source on SCP production was studied by formulating Fermentation media with bagasse extract and fish waste hydrolysate. Fermentation media containing inorganic salts and sugarcane bagasse extract (20%, 100%), fish waste hydrolysate (50%, 100%) were used for SCP production (Media composition (200ml) : (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>- 1g, KH<sub>2</sub>PO<sub>4</sub>- 0.2g, MgSO<sub>4</sub>- 0.1g, NaCl- 0.02g, CaCl<sub>2</sub>- 0.02g, pH- 6.2). Role of minerals in SCP production was checked by formulating media containing only 20% sugarcane bagasse extract and 50% fish waste hydrolysate.

Combined effect on C & N source on SCP production was also analysed by formulating media which was as follows: a) For *Bacillus clausii*: 100ml bagasse extract + 100ml fish waste hydrolysate + inorganic salts, For *Lactobacillus*: 40ml bagasse extract + 160ml fish waste hydrolysate + inorganic salts.

Post fermentation, Bacterial cells were separated by centrifugation at 20,000 RPM for 20 minutes. The obtained bacterial pellet was dried at 60°C until consistent dry weight was obtained. The pellet was further dried at 110°C for 1 hour. The protein quantitation of the bacterial pellet was done by Folin-Lowry analysis. Anthrone analysis was carried out for total carbohydrate quantitation.

### III. RESULTS

#### 3.1 Enrichment, isolation & identification of *Lactobacillus* & *Bacillus clausii*

*Lactobacillus* species & *Bacillus clausii* culture was enriched & isolated on MRS and Nutrient medium respectively (Fig. 1 a, c). Morphology & Gram nature was determined by Gram staining technique (Fig. 1 b, d). Confirmation of genus was done by performing Biochemical tests (Table 1).

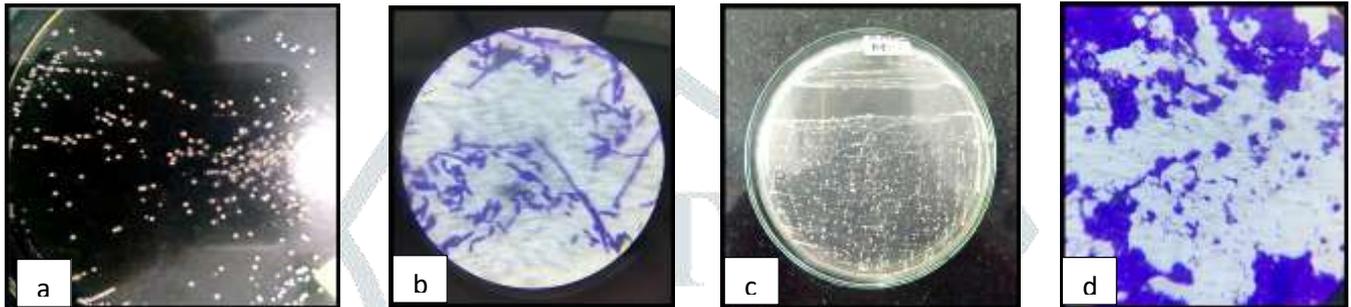


Fig. 1. Isolation and Gram staining of *Lactobacillus* species & *Bacillus clausii*

a. *Lactobacillus* species on MRS agar showing off white coloured colonies b. Gram positive bacilli of *Lactobacillus* (1000x), c. *Bacillus clausii* on nutrient agar, d. Gram positive short rods of *Bacillus clausii* (1000x)

Table 1. Biochemical test results of *Lactobacillus* and *Bacillus clausii*

Test	<i>Lactobacillus</i>	<i>Bacillus clausii</i>
Sugar fermentation		
a. Glucose	+	+
b. Lactose	+	-
c. Xylose	-	+
d. Mannitol	-	-
e. Sucrose	+	-
f. maltose	+	-
Oxidative/fermentative test		
a. aerobic	Acid production	Acid production
b. anaerobic	Acid production	No change
Catalase test	Negative	Positive
Oxidase test	Negative	Positive
Casein digestion test	Growth observed, no zone of clearance	Growth observed, no zone of clearance
Starch hydrolysis test	NA	Negative
Nitrate reductase test	NA	Negative

key: (+) – acid production, (-) – no acid production, (+\*) – acid and gas production

#### 3.2 Hydrolysis, sugar & protein quantitation of substrate

Sugarcane bagasse and fish waste from Surmai (*Scomberomorus guttatus*) were used as substrates to provide carbohydrate and nitrogen source. The total reducing sugars in the sugarcane bagasse hydrolysate were found to be 2.696g/L by the DNSA technique. The total protein content in the separated liquid fraction of fish viscera hydrolysate was estimated to be 19.18g/L.

### 3.3 Optimisation of fermentable sugar concentration

High concentration of sugar can inhibit growth of microorganisms. Thus, it is important to optimize sugar concentration before setting up fermentation. Minimum inhibitory concentration (MIC) of fermentable sugar was determined using broth dilution technique & it was found to be 30% for *Lactobacillus* (Fig. 2 a) & *Bacillus clausii* (Fig. 2 b). Hence, 20% carbohydrate concentration was selected for fermentation purpose.

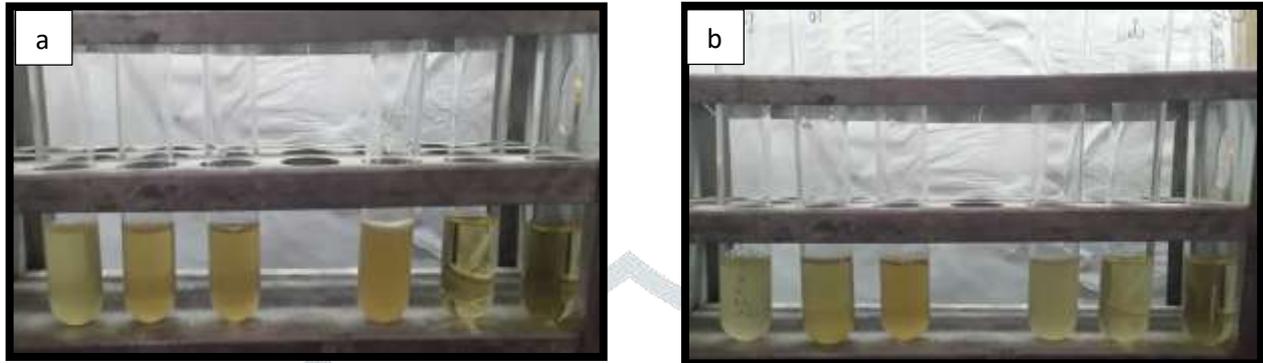


Figure 2: MIC determination of sugar (a. *Lactobacillus* b. *Bacillus clausii*)

### 3.4 SCP production

Sugarcane baggase extract and Fish waste hydrolysate were used as C & N source for SCP production. Submerged batch fermentation was performed at 28°C for 1 week (Fig. 3 a, b). Combined effect of C & N source on SCP production was also studied by formulating media containing optimum concentration of Sugarcane baggase extract and Fish waste hydrolysate. Maximum biomass was observed when both *Lactobacillus* & *Bacillus clausii* were cultured in baggase extract medium (5.088 g/L & 1.990 g/L respectively) compared to Synthetic & fish waste hydrolysate medium. When mixed fermentation was performed using both C & N sources (i.e. Sugarcane baggase extract and Fish waste hydrolysate), biomass production was increased two fold (10.640 g/L) for *Lactobacillus* whereas for *Bacillus clausii* 8 fold (8.430 g/L) increase was reported.

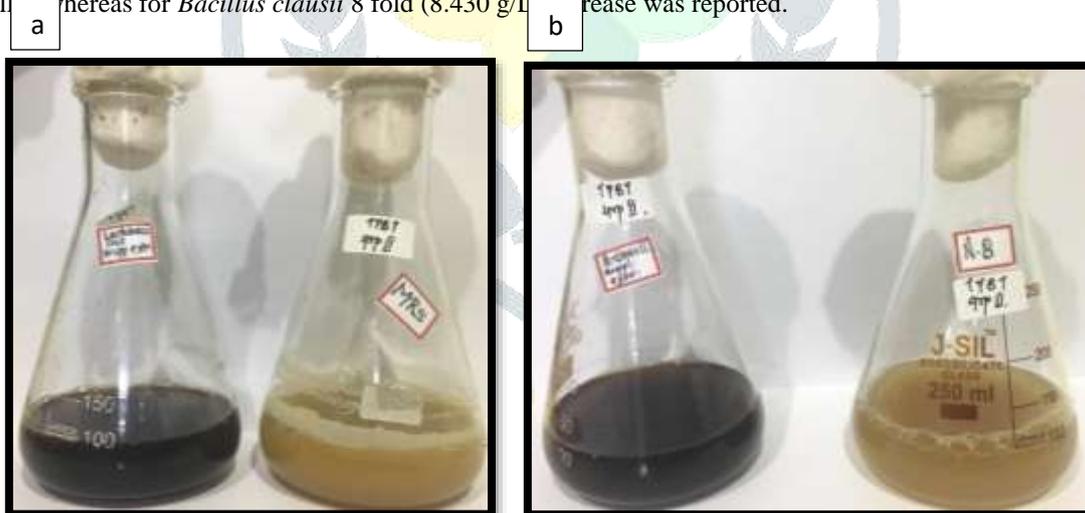
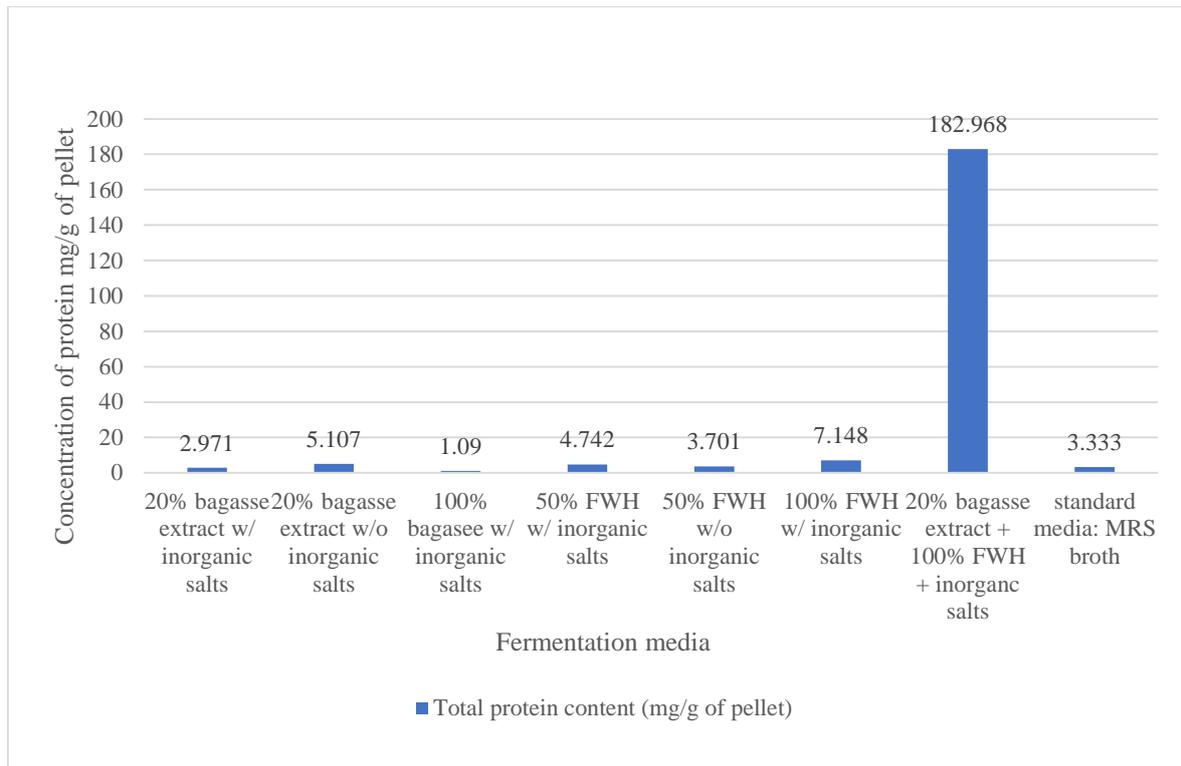


Figure 3: SCP production using Synthetic media & Mineral medium supplemented with C & N source (a. *Lactobacillus* b. *Bacillus clausii*)

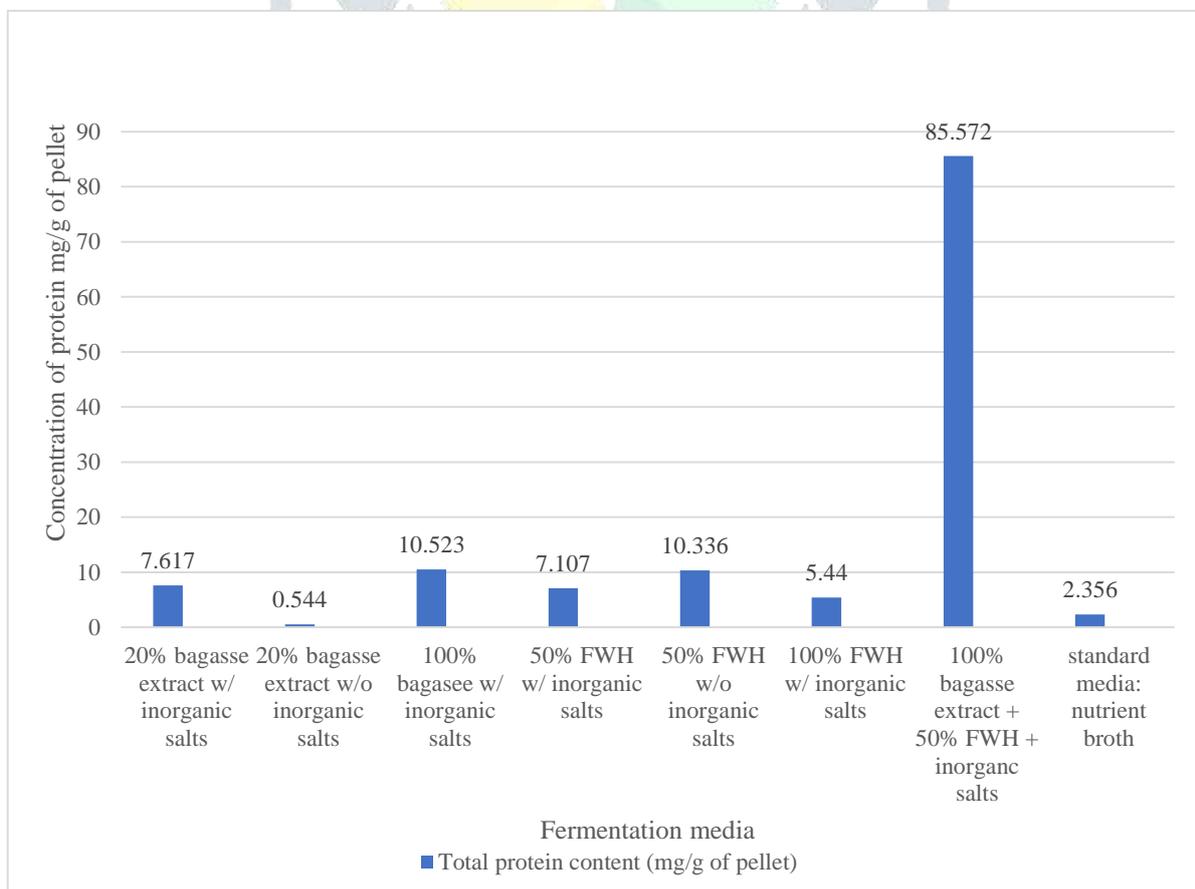
The effect of nutrient supplements in SCP production is shown in Graph 1, 2. Results indicate that higher protein production was found in higher biomass when both cultures were grown in Baggase extract + Fish waste hydrolysate + inorganic salts containing medium (Graph 1, 2), 182.96 mg/g & 85.57 mg/g for *Lactobacillus* & *Bacillus clausii* respectively. It suggests that both substrates together provide all nutrients required for higher protein production. Compared with SCP production carried out by other organisms this yield is low and it can be due to less amount of sugar & nitrogen supplements were present in sugarcane baggase & Fish waste hydrolysate. This low amount resulted in less biomass thus decreased SCP production.

Above all results indicate that *Lactobacillus* & *Bacillus clausii* were able to grow on Baggase extract & Fish waste hydrolysate medium individually but it results in lower biomass & consequently less SCP production. When both sources in their optimum concentration were used for fermentation, it resulted in comparatively higher protein production.

Graph 1: Total protein estimation of *Lactobacillus* species grown in different fermentation media



Graph 2: Total protein estimation of *Bacillus clausii* grown in different fermentation media



#### IV. CONCLUSION

SCP production from *Lactobacillus* & *Bacillus clausii* was carried out by submerged fermentation using Agricultural (Sugarcane bagasse extract) & Animal waste (Fish waste hydrolysate). The chemical hydrolysis of bagasse yields about, 2.596g/L reducing sugars which was employed as fermentable carbohydrate source for fermentation. The enzymatic digestion of fish waste obtained from Surmai fish gave 19.18g/L of proteins which were then used as nitrogen source for formulation of fermentation media.

Biomass & SCP production can be increased if *Lactobacillus* & *Bacillus clausii* were grown in fermentation media supplemented with both bagasse extract & fish waste hydrolysate. The highest amount of protein production in *Lactobacillus* was of 182.96mg/1g of pellet in the media containing both fish waste hydrolysate and sugarcane bagasse extract as well as inorganic salts. *Bacillus clausii* as well showed highest protein production in media containing fish waste hydrolysate and sugarcane bagasse extract and inorganic salts. It was about 85.57mg/1g of pellet. It was observed that both the bacterial species show greater extent of growth at room temperature rather than 37 degree Celsius. Similarly, the bacteria show comparative more growth at agitation conditions rather than static conditions.

When compared to SCP production of standard media (3.33 mg / gm in MRS medium) the *Lactobacillus* gave about 54 times higher protein content in the formulated fermentation media. Similarly, *Bacillus clausii* gave 36 times more protein concentration than that of standard synthetic media (2.36 mg / gm in nutrient broth). Overall, *Lactobacillus* species is better at producing high amounts of protein than that of *Bacillus clausii*.

The present finding reveals that only Sugarcane bagasse extract & Fish waste hydrolysate are not sufficient to enhance biomass as well as SCP production. SCP content can be enhanced by incorporating other substrates rich in sugar such as Glucose which may help to increase biomass & subsequently high SCP. Thus, Agricultural and Animal waste such as sugarcane bagasse extract & fish waste hydrolysate can be used as potential substrate for production of cellular biomass of edible bacteria.

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