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Cost effective production of *Bacillus thuringiensis* subsp. *israelensis* using chemically pre-treated rice straw

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Abstract

Bacillus thuringiensis subsp. *israelensis* is the leading biopesticide in the world due to its specificity and lack of toxicity. The use of *Bti* is still restricted in developing countries due to the bioprocess complications and high cost of production. In the present study we have shown that lignocellulosic agro waste residues like rice straw can be used as cost effective substrates with appropriate chemical treatment methods. We have shown that compared to untreated rice straw, pre-treated rice straw gives better biomass production, and also shows less sporulation time, which in turn gives reduced larval killing time as part of larvicidal assay. Sodium hydroxide (NaOH) has proven to be a better delignifying agent compared to Calcium hydroxide (CaOH). It is found that supplementation of the medium with the fish slurry enhances biomass production in the case of both NaOH and CaOH. This biomass enhancement is also visible when chicken feather hydrolysate is used as Nitrogen supplement, although it is not as effective as fish hydrolysate.

Keywords: *Bacillus thuringiensis* subsp. *israelensis*, biopesticide, biomass, rice straw, delignification, nitrogen supplementation

1. Introduction

Mosquitoes cause more human suffering than any other organism. Over one million people worldwide die from mosquito-borne diseases every year. Every year there are more than 700,000 deaths which occurs globally from diseases such as Malaria, Dengue, Schistosomiasis, Human African trypanosomiasis, Leishmaniasis, Chagas disease, Yellow fever, Japanese encephalitis and Onchocerciasis.

In 2020, globally there was an estimated 243 million Malaria cases with 8,63,000 deaths. 89% of the reported deaths were in Africa. Most of the deaths were among children under 5 years' old and pregnant women. The annual economic cost of Malaria in Africa in terms of foregone production have been estimated to be about US \$12billion. Thus, it is very much necessary to control mosquitoes.

As vectors thrive under conditions where people are poor, water is unsafe and environment is contaminated with filth, these diseases extract their heaviest toll on the poor – the people left behind by development. Measure that controls the vectors, the agents of disease, provide an excellent, but underutilized opportunity to help these people come up. Usually, children are more prone to being infected due to various diseases transmitted by flies, mosquitoes and other insects which act as vectors.

Mosquito larval control is a key component of integrated mosquito management control. Many strategies have been used to control them. One strategy is the use of chemical pesticides with a broad spectrum of activity. However, there are a number of disadvantages to using such chemical pesticides [1]. The chemical insecticides are responsible for the loss of natural predators and for the presence of insecticide residues in our food chain. In some cases, it has even led to highly detrimental bio amplification.

In recent years, the use of biological agents for controlling mosquitoes has rapidly increased. Harmless nature of bioinsecticide products makes them attractive for application in urban as well as rural areas.

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However, these bioinsecticides have not lived up to the expectations of becoming key players in the global market. The commercial introduction of new products is hindered by the time and financial investment required for basic research and product development, registration and commercialization. Bacterial larvicides which produce toxins lethal to the larvae of *Diptera* are very specific, resulting in lower environmental impact and thus are preferred in natural habitats [2]. For the past 40 years, numerable strains of *Bacillus thuringiensis* have been in use. *Bt* has advantages compared to chemical insecticides in being very selective, narrow spectrum, so it is very safe to other species [3]. However, *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) is the most widely adopted strain. Since *Bti* has multi protein toxin complex, the possibility of insect developing resistance is very low [4]. It is the most effective bio-larvicide against mosquitoes that is available to date [5-11]. *Bti* is a gram positive, rod shaped, facultative aerobic, spore forming bacterium having genome size of 2.4 to 5.7 million base pairs. The prevalence of this strain is not restricted to soil but has been isolated worldwide from many types of habitats [12]. It produces four different crystal inclusions formed by Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa and is reported to be highly toxic against *Aedes aegypti* and *Culex sp.* species. Cry4 protein toxin genes and Cyt genes are *Dipteran* specific. All these toxins show synergistic effect to overcome the insect resistance [13-15]. The ingestion by mosquito larva leads to solubilization of the *Bti* parasporal body in the alkaline gut juices. The protoxin is cleaved by midgut proteases to form active δ -endotoxin proteins. These active endotoxins damage the midgut wall by disrupting the function of midgut epithelial cell membranes by binding to their receptors.

In this study we have attempted to develop a cost-effective process using pre-treated rice straw for the production of *Bacillus thuringiensis* subsp. *israelensis* H-14 based biopesticides. As part of the study, we have experimented with different pre-treatment techniques like delignification of rice straw using Sodium hydroxide (NaOH), Calcium hydroxide (CaOH).

Since rice straw is lignocellulosic waste with high carbon and less nitrogen, further experiments were done to supplement pre-treated rice straw with fish slurry and poultry feather hydrolysate.

Rice straw contains cellulose, hemicellulose and lignin. Out of these, lignin is a polyphenolic material and it encases the cellulose, hemicellulose fibre's present in the rice straw. So, there is a technical requirement for disrupting the lignin sheath. This was done by using alkaline materials like Sodium hydroxide (NaOH), Calcium hydroxide (CaOH).

India is having one of the longest coastlines in the world, with the length of approximately 7,000 km. out of the total catch, almost 30% goes as waste and these massive amounts of rotten fish can be channelized for the production of *Bti* based biopesticide by supplementing much required nitrogen source. Similarly, poultry feather is a by-product of poultry meat consumption which contains keratin protein. This can be pre-treated to be converted as peptides and amino acids and utilized as a cost-effective nitrogen supplement for the large-scale production of *Bacillus thuringiensis* subsp. *israelensis*.

2. Materials and Methods

2.1 Bacterial culture and maintenance

Bacterium used in this study was serotype H-14 of *Bacillus*

thuringiensis subsp. *israelensis*. The bacteria was grown in modified Glucose Yeast Extract salt (mGYS) broth, containing glucose (0.3%), Ammonium sulphate (0.2%), Yeast Extract (0.2%), Dipotassium hydrogen phosphate (0.5%), Magnesium sulphate (0.02%), Calcium phosphate (0.008%) at pH of 7.2 and temperature of 30 °C. To avoid clumping of cells, all cultures were passed through a pre-culture step.

2.1.2 Preculture stage

In all cases, the cultivation of bacteria began with a preculture stage. A loop full of the refrigerated preserved culture, was transferred to 20 ml of mGYS broth in 100 ml flask and incubated stagnant for 12-15 hours. For further cultivation, 1ml of the preculture was used as the inoculum for 100 ml of the medium.

2.2 The production of *Bti* based biopesticide using Rice straw with agar-based media

2.2.1 Materials

Rice straw (RS) powder, Peptone, Yeast Extract, Agar

2.2.2 Procedure

Different solid media were prepared in a) 1% Rice straw alone, b) 1% RS, 0.1% Peptone, Agar, c) 1% RS, 0.1% Peptone, 0.1% Yeast Extract, 2% Agar. 0.1ml of *Bti* preculture was prepared and inoculated into the respective petriplates containing the three different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

2.3 The production of *Bti* based biopesticide using chemically delignified (NaOH) straw based medium with agar.

2.3.1 Materials

Rice straw, Peptone, Yeast Extract, Sodium hydroxide, Agar

2.3.2 Procedure

Different solid media were prepared with a) 1% Delignified rice straw powder, 2% Agar, b) 1% Delignified rice straw powder, 0.1% Peptone, 2% Agar, c) 1% Delignified rice straw powder, 0.1% Peptone, 0.1% Yeast Extract, 2% Agar

1N NaOH was used to delignify rice straw and the resulting delignified rice straw was used for further experiments. 10g straw was dipped in 100 ml NaOH solution for 1 hour and the supernatant was discarded. The residue was washed with distilled water. The residue was resuspended in 100 ml distilled water, supplemented with 100 mg Yeast Extract and 100 g Peptone. The pH was adjusted to 7.2, then 2g agar was also added. It was then autoclaved for 15 minutes at 15 psi at 121 °C. After cooling to room temperature, poured into plates and it was inoculated with 0.1ml of *Bti* preculture and allowed to grow.

0.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing three different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

2.4 The production of *Bti* based biopesticide using chemically delignified (CaOH) straw based medium with agar

2.4.1 Materials

Rice straw, Peptone, Yeast Extract, Calcium hydroxide, Agar

2.4.2 Procedure

Different Solid media were prepared with a) 1% Delignified rice straw powder, 2% agar, b) 1% Delignified rice straw powder, 0.1% Peptone, 2% agar, c) 1% Delignified rice straw powder, 0.1% Peptone, 0.1% Yeast Extract, 2% agar.

1N CaOH was used to delignify it and the resulting delignified straw was used for further experiments. 10g straw was dipped in 100 ml CaOH solution for 1 hour and the supernatant was discarded. The residue was washed with distilled water. The residue was resuspended in 100 ml distilled water, supplemented with 100 mg Yeast Extract and 100g Peptone. The pH was adjusted to 7.2, then 2g agar was also added, then it was autoclaved for 15 minutes at 15 psi at 121 °C. After cooling to room temperature, 0.1 ml *Bti* preculture was prepared and inoculated into the respective petriplates containing the three different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

2.5 Preparation of fish slurry

100 g of Sardine was taken and its head, scales and visceral contents were removed. Remaining tissue was steamed for five minutes in a pressure cooker. Fish bone was removed after cooking and from resultant tissue 5g was suspended in 100 ml water and minced well

2.5.1 The production of *Bti* based biopesticide using delignified rice straw with NaOH agar based medium supplemented with fish slurry

Delignification of rice straw was done as described earlier mentioned in section 2.4. 10 g residue was resuspended in 100 ml distilled water and supplemented with 0.1, 0.2, 0.3, 0.4, and 0.5 ml of fish slurry and 100 mg Yeast Extract. pH was adjusted to 7.2, then 2g agar was also added, it was then autoclaved for 15 minutes at 15 psi and 121 °C. After cooling to room temperature, it was poured into petri plates and was inoculated with 0.1ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

2.5.2 The production of *Bti* based biopesticide using delignified rice straw with CaOH agar based medium supplemented with fish slurry

Delignification of rice straw was done as described earlier mentioned in section 2.4. 10g residue was resuspended in 100 ml distilled water supplemented with 0.1, 0.2, 0.3, 0.4, and 0.5 ml of fish slurry and 100 mg of Yeast Extract. pH was adjusted to 7.2, then 2g agar was added into it. It was then

autoclaved for 15 minutes at 15 psi and 121 °C. After cooling to room temperature, poured into plates and it was inoculated with 0.1ml of *Bti* preculture and allowed to grow for 48 hours.

2.6 Supplementation with chicken feather hydrolysate

2.6.1 Preparation of chicken feather hydrolysate

The chicken feathers were collected from poultry farms and washed thrice to remove dirt and other debris like blood. It was subjected to treatment with Chloroform: Methanol (1: 1 v/v) for 12 hours. The resulting defatted feathers were filtered and dried in sun light. Then it was powdered and the subjected to 0.1N NaOH treatment at 90 °C for 30 minutes. 1g from this was used for hydrolysis. After hydrolysis the resulting material is filtered and the filtrate was used for further studies.

2.6.2 The production of *Bti* based biopesticide using Chemically delignified rice straw with NaOH, supplemented with chicken feather hydrolysate

Delignification was done with 1. N Sodium hydroxide as in 2.3. 10 g of rice straw was dipped in 100 ml of NaOH for 1 hour, and the supernatant was discarded. The residue was washed with distilled water. 1g residue was resuspended in 100ml distilled water supplemented with 0.1ml, 0.2ml, 0.3ml, 0.4. ml and 0.5ml of chicken feather hydrolysate and 100 mg of Yeast Extract. Then the pH was adjusted to 7.2. Then it was autoclaved for 15 minutes at 15 psi at 121 °C. After cooling to room temperature, it was inoculated with 0.1 ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

2.6.3 The production of *Bti* based biopesticide using Chemically delignified rice straw with CaOH supplemented with chicken feather hydrolysate

The experiment was done as described earlier in section 2.5.2. The biomass concentration, sporulation status and larvicidal activity were determined

3. Results

3.1 Batch fermentations of *Bti* using Rice straw as the growth media

Table 1 shows the data of *Bacillus thuringiensis* subspecies *israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using rice straw as the main raw material (carbon source) and Peptone and Yeast Extract as medium supplements.

Table 1: Biomass yield, sporulation timings and bioassay data of *Bti* when grown in SSF using rice straw

Solid State Fermentation (Ssf)			
Media composition	Wet weight (g) per 100ml of media (SD ± 10%)	Sporulation time in hour (SD ± 0.25 hour)	Bioassay killing time in SSF in minute (SD ± 15 minute)
1% Rice straw powder (RS) + 2% Agar	0.06	67	480
1% RS + 0.1% Peptone + 2% Agar	0.062	55	378
1% RS + 0.1% Peptone + 0.1% Yeast Extract + 0.2% Agar	0.0622	47	320

3.2 Batch fermentations of *Bti* using delignified rice straw with NaOH as the growth media

Table 2 shows the data of *Bacillus thuringiensis* subspecies *israelensis* biomass yield, sporulation timings and bioassay

data when grown in SSF using delignified (using Sodium hydroxide) rice straw as the main raw material (carbon source) and Peptone and Yeast Extract as medium supplements.

Table 2: Biomass yield, sporulation timings and bioassay data of *Bti* grown in SSF using rice straw delignified by Sodium hydroxide

Solid State Fermentation (SSF)			
Media composition	Wet weight (g) per 100ml of media (SD \pm 10%)	Sporulation time in hour (SD \pm 0.25 hour)	Bioassay killing time in SSF in minute (SD \pm 15 minute)
1% Delignified Rice straw (DRS) powder +2% Agar	0.38	55	360
1% Delignified rice straw powder + 0.1% Peptone + 2% Agar	0.42	43	290
1% Delignified rice straw powder + 0.1% Peptone + 0.1% Yeast Extract + 2% Agar	0.45	38	230

3.3 Batch fermentations of *Bti* using delignified Rice straw with CaOH as the growth media

Table 3 shows the data of *Bacillus thuringiensis* subspecies *israelensis* biomass yield, sporulation timings and bioassay

data when grown in SSF using delignified (using Calcium hydroxide) rice straw as the main raw material (carbon source) and Peptone and Yeast Extract as medium supplements.

Table 3: Biomass yield, sporulation timings and bioassay data when grown in SSF using rice straw delignified using Calcium hydroxide with Peptone and Yeast Extract as medium supplements.

Solid state fermentation (SSF)			
Media composition	Wet weight (g) per 100ml of media (SD \pm 10%)	Sporulation time in hour (SD \pm 0.25 hour)	Bioassay killing time in SSF in minute (SD \pm 15 minute)
1% Delignified Rice straw powder + 2% Agar	0.358	56	370
1% Delignified rice straw powder + 0.1% Peptone + 2% Agar	0.38	45	321
1% Delignified rice straw powder + 0.1% Peptone + 0.1% Yeast Extract + 2% Agar	0.446	36	245

3.4.1 Batch fermentations of *Bti* using delignified rice straw with NaOH supplemented with fish slurry

Table 4 shows the data of *Bacillus thuringiensis* subspecies *israelensis* biomass yield, sporulation timings and bioassay

data when grown in SSF using delignified (using Sodium hydroxide) rice straw as the main raw material (carbon source) and fish slurry and Yeast Extract as medium supplements.

Table 4: Biomass yield, sporulation timings and bioassay data when grown in SSF using rice straw delignified using Sodium hydroxide with fish slurry and Yeast Extract as medium supplements.

Solid State Fermentation (SSF)			
Media composition	Wet weight (g) per 100ml of media (SD \pm 10%)	Sporulation time in hour (SD \pm 0.25 hour)	Bioassay killing time in SSF in minute (SD \pm 15 minute)
1% Delignified Rice straw powder + 0.1ml Fish slurry (FS) + 0.1% Yeast extract+2% Agar	0.452	49	222
1% Delignified rice straw powder + 0.2ml FS + 0.1% Yeast Extract + 2% Agar	0.534	44	210
1% Delignified rice straw powder + 0.3ml FS + 0.1% Yeast Extract + 2% Agar	0.57	42	200
1% Delignified rice straw powder + 0.4ml FS + 0.1% Yeast Extract +2% Agar	0.584	36 -38	213
1% Delignified rice straw powder + 0.5ml FS + 0.1% Yeast Extract + 2% Agar	0.546	41	219

3.4.2 Batch fermentations of *Bti* using delignified rice straw with CaOH supplemented with fish slurry

Table 5 shows the data of *Bacillus thuringiensis* subspecies *israelensis* biomass yield, sporulation timings and bioassay

data when grown in SSF using delignified (using Calcium hydroxide) rice straw as the main raw material (carbon source) and fish slurry and Yeast Extract as medium supplements.

Table 5: Biomass yield, sporulation timings and bioassay data when grown in SSF using rice straw delignified using Calcium hydroxide with fish slurry and Yeast Extract as medium supplements.

Solid State Fermentation (SSF)			
Media composition	Wet weight (g) per 100ml of media (SD \pm 10%)	Sporulation time in hour (SD \pm 0.25 hour)	Bioassay killing time in SSF in minute (SD \pm 15 minute)
1% Delignified Rice straw powder + 0.1ml Fish slurry + 0.1% Yeast Extract + 2% Agar	0.378	44	240
1% Delignified rice straw powder + 0.2ml Fish slurry +0.1% Yeast Extract + 2% Agar	0.388	40	235
1% Delignified rice straw powder + 0.3ml Fish slurry + 0.1% Yeast Extract + 2% Agar	0.452	37	231
1% Delignified rice straw powder + 0.4ml Fish slurry + 0.1g Yeast Extract + 2% Agar	0.448	39	236
1% DRS+ 0.5ml Fish slurry + 0.1g Yeast Extract + 2% Agar	0.446	38	243

3.5.1 Batch fermentations of *Bti* using delignified rice straw with NaOH supplemented with chicken feather hydrolysate

Table 6 shows the data of *Bacillus thuringiensis* subspecies *israelensis* biomass yield, sporulation timings and bioassay

data when grown in SSF using delignified (using Sodium hydroxide) rice straw as the main raw material (carbon source) and chicken feather hydrolysate Yeast Extract as medium supplements.

Table 6: Biomass yield, sporulation timings and bioassay data when grown in SSF using rice straw delignified using Sodium hydroxide with chicken feather hydrolysate Yeast Extract as medium supplements.

Solid state fermentation (SSF)			
Media composition	Wet weight (g) per 100ml of media (SD ± 10%)	Sporulation time in hour (SD ± 0.25 hour)	Bioassay killing time in SSF in minute (SD ± 15 minute)
1g Delignified rice straw powder + 0.1ml CFH + 0.1g Yeast Extract + 2% Agar	0.358	47	232
1g Delignified rice straw powder + 0.2ml CFH + 0.1g Yeast Extract + 2% Agar	0.378	44	220
1g Delignified rice straw powder + 0.3ml CFH + 0.1g Yeast Extract + 2% Agar	0.386	39	214
1g Delignified rice straw powder + 0.4ml CFH + 0.1g Yeast Extract + 2% Agar	0.374	47	221
1g Delignified rice straw powder + 0.5ml CFH + 0.1g Yeast Extract + 2% Agar	0.354	46	217

3.5.2 Batch fermentations of *Bti* using delignified rice straw with CaOH supplemented with chicken feather hydrolysate

Table 7 shows the data of *Bacillus thuringiensis* subspecies *israelensis* biomass yield, sporulation timings and bioassay

data when grown in SSF using delignified (using Calcium hydroxide) rice straw as the main raw material (carbon source) and chicken feather hydrolysate and Yeast Extract as medium supplements.

Table 7: Biomass yield, sporulation timings and bioassay data when grown in SSF using rice straw delignified using Calcium hydroxide with chicken feather hydrolysate and Yeast Extract as medium supplements.

Solid State Fermentation (SSF)			
Media composition	Wet weight (g) per 100ml of media (SD ± 10%)	Sporulation time in hour (SD ± 0.25 hour)	Bioassay killing time in SSF in minute (SD ± 15 minute)
1g DRS + 0.1ml CFH + 0.1g Yeast Extract + 2% Agar	0.352	43	247
1 DRS + 0.2ml CFH + 0.1g Yeast Extract + 2% Agar	0.366	39	238
1 DRS+ 0.3ml CFH + 0.1g Yeast Extract + 2% Agar	0.426	39	234
1 DRS+ 0.4ml CFH + 0.1g Yeast Extract + 2% Agar	0.422	37	239
1 DRS+ 0.5ml CFH + 0.1g Yeast Extract + 2% Agar	0.382	39	243

4. Discussion

4.1 Batch fermentations of *Bti* using Rice straw as the growth media

Table 1 shows the growth of *Bti* using rice straw as the main carbon source media. It was found that raw untreated rice straw gives negligible biomass output. In addition, even supplementation with Peptone and Yeast Extract etc. also didn't improve the biomass output to be viable in an economical way. The reason for this can be attributed to the unique biochemical structure of the rice straw and the inability of *Bti* to secrete sufficient quantities of extracellular hydrolytic enzymes necessary for assimilating the biopolymers inherent in the rice straw. With raw rice straw only the biomass output was 0.03g, with supplementation of Peptone 0.0315g and by additional supplementation of Yeast Extract it was 0.0311g.

Rice straw (RS) is unique in its structure due to the presence and peculiar arrangement of cellulose (40%) hemicellulose (26%) and lignin (18%).

India being the second largest producer of rice straw after China. India has 30,000 varieties of paddy [16]. The RS cell walls constitute three biopolymers, namely, cellulose, hemicellulose, and lignin. Cellulose is a homopolymer of cellobiose, which are linked by -1, 4-glycosidic bonds. The complete hydrolysis of cellulose yields glucose, which is a preferable carbon source for commonly used fermenting microorganisms in industry. Hemicellulose contains a mixture of polysaccharides by which Xylan represents a major portion. Xylan consists of xylose units, which are linked by -1, 4-glycosidic bonds to form Xylan main chain. Typically, the main chain is substituted with some sugars (i.e., arabinose, galactose, and glucose), sugar acids (i.e., glucuronic acid), and ferulic and *p*-coumaric acids, depending on the source of

Xylan's. Lignin is a large complex polymer of unrepeatable phenolic monomers. It significantly contributes to the water conduction and defense systems in plants. However, the hydrophobicity and complex structure (also heterogeneity) of lignin pose challenges for biomass processing and utilization. Since *Bti* is a very poor producer of cellulase enzyme, the results generated tallies [17].

4.2 Batch fermentations of *Bti* using Delignified rice straw with NaOH, as the growth media.

Compared to other pretreatment methods, alkali pretreatments are the most effective ones because of its ability to increase cellulose accessibility and lignin removal capability. Alkali pretreatments work on the principle of saponification (de-esterification) of Xylan-hemicellulose and lignin intermolecular crosslinking ester bonds. As much as the crosslinks (bonds) removed, lignocellulose porosity increased. When NaOH is used as a pretreatment process, the internal surface area increases and crystallinity and degree of crystallinity are increased due to the swelling of lignocellulose material caused by NaOH that eventually leads to the removal of amorphous parts.

Sodium hydroxide, also known as lye and caustic soda, is an inorganic compound with the formula NaOH. It is a white solid ionic compound consisting of Sodium cations (Na⁺) and hydroxide anions (OH⁻).

Sodium hydroxide is a strong alkali that hydrolyses proteins and carbohydrates at ordinary ambient temperatures and may cause severe chemical burns. It is highly soluble in water, and readily absorbs moisture and carbon dioxide from the air.

Sodium hydroxide is used in many industries: In the manufacture of pulp and paper, textiles, drinking water, soaps and detergents, and as a drain cleaner. Worldwide production

in 2018 was approximately 60 million tons, while demand was 51 million tons. Since it is a cheap compound, it can be used very cost effectively for the large-scale production of *Bti* after proper hydrolyzing the rice straw derived cellulose and using the resulting glucose as carbon source.

Since *Bti* is a poor producer of cellulase enzyme and due to the highly complicated structure of lignocellulosic structure of rice straw, Chemical hydrolysis with alkali, (NaOH) was attempted. Alkaline hydrolysis will break the lignin outer coat resulting in delignification and more over the exposed cellulose microfibrils will be digested /cleaved to release glucose monomers. The growth and biomass production clearly shows the enhanced biomass output using delignified rice straw.

Biomass output was 0.19 g with 1% delignified rice straw, compared to raw rice straw 0.03 g and with supplementations (1%DRS+0.1% Peptone, 1% Yeast Extract) biomass output was 0.225 g compared to 0.0311g. The bio larvicidal assay time achieved was 230 minutes. This clearly proves that chemical delignification of rice straw is a pre-requisite for enhanced *Bti* production from rice straw as the substrate.

4.3 Batch fermentations of *Bti* using delignified rice straw with CaOH, as the growth media

Calcium hydroxide is an inorganic compound with the chemical formula CaOH. It is a colorless crystal or white powder and is produced when quicklime (Calcium oxide) is mixed, or slaked with water. It has many names including hydrated lime, caustic lime, builders' lime, slack lime, Calcium, or pickling lime. Calcium hydroxide is used in many applications, including food preparation. Lime water is the common name for a saturated solution of Calcium hydroxide. Since it is cheaper than Sodium hydroxide and easily available in bulk quantities there is possibility of using this chemical for hydrolysis of straw and production of *Bti* in a cost-effective manner, although there is a slight drop in biomass production.

Delignification of rice straw was done using mild alkaline Calcium hydroxide. Sodium hydroxide being a strong alkali will create environmental pollution when the process is scaled up. As a solution to this Calcium hydroxide which is less polluting can be used. Table 3 gives the biomass output data with delignified rice straw powder (1%) and with supplementations of Peptone and Yeast Extract which are 0.179 and 0.223 g respectively. The culture has been found to sporulate in 46 hours which is much lesser compared to control (48 hours). Bioassays related killing time is 245 minutes which proves high potency.

4.4.1. Batch fermentations of *Bti* using delignified rice straw with NaOH, supplemented with fish slurry

C: N ratio of rice straw is 80:1. So, there is need to incorporate Nitrogen in to the medium in order to satisfy the microbial requirement. In this context fish is found to be a cheap medium supplement, considering the total quantity of fish wastes available in the country.

Fishing in India is a major industry in its coastal states, employing over 14 million people. In 2016-17, the country exported 11, 34, 948 metric tons of sea food worth US\$5.78 billion (₹ 37, 870.90 crore), frozen shrimp being the top item of export. According to the Food and Agriculture Organization (FAO) of the United Nations, fish production has increased more than tenfold since 1947.

India has 8, 129 kilometers (5, 051 mi) of marine coastline, 3, 827 fishing villages and 1, 914 traditional fish landing centers. India's fresh water resources consist of 195, 210 kilometers (121, 300 mi) of rivers and canals, 2.9 million hectares of minor and major reservoirs, 2.4 million hectares of ponds and lakes, and about 0.8 million hectares of flood plain wetlands and water bodies.

As per Table.4 chemically delignified rice straw (NaOH) with various concentrations of fish slurry and with Yeast Extract supplementation, the biomass output was 0.226, 0.267, 0.285, 0.292, 0.293 (with 0.1ml, 0.2, 0.3, 0.4and 0.5 fish slurry) 0.4 ml was found to be optimum which gives maximum output, the control was having 0.225g with Peptone. Sporulation timing was in the range of 36-38 hours. and killing time was 213 minutes. This has conclusively proved that supplementation of fish slurry will greatly enhance yield and profitability of this biopesticide.

4.4.2 Batch fermentations of *Bti* using delignified rice straw with CaOH, supplemented with fish slurry

As per Table 5 chemically delignified rice straw (CaOH) with various concentrations of fish slurry and with Yeast Extract supplementation, the biomass output was 0.189, 0.194, 0.226, 0.224, 0.223 (with 0.1ml, 0.2, 0.3, 0.4and 0.5 fish slurry) 0.4 ml was found to be optimum which gives maximum output, the control was having 0.223. g with Peptone. This is really cost effective when a comparison of the price of Peptone and fish slurry cost is done. Sporulation timing was 39 hours. and killing time was 236 minutes. This has conclusively proved that supplementation of fish slurry will greatly enhance yield and profitability of this biopesticide.

4.5.1 Batch fermentations of *Bti* using delignified rice straw with NaOH, supplemented with chicken feather hydrolysate

The poultry feather is the most important waste by-product resulting from poultry processing plants, reaching billions of tons annually worldwide. Specifically, the Tunisian contribution is about 20, 000 tons. This by-product presents 10% of the total weight of poultry. The feather is a rich protein source because it is the most abundant keratinous protein in nature, representing 80–90% of the total composition of the feather. Keratin has a high stable mechanical structure attributed to the high degree of cross-linking by disulfide bonds, hydrogen bonds and hydrophobic interactions.

Table 6 shows the batch fermentations of *Bti* using delignified rice straw (NaOH) supplemented with chicken feather hydrolysate. Like fish hydrolysate chicken or poultry feather hydrolysate is also a rich source of various amino acids. Since the cost of Peptone is exorbitant, *Bti* biomass production using chicken feather hydrolysate is a cost-effective method to manufacture this highly sought after biopesticide.

Table 6 shows the biomass, sporulation time and larvicidal assay killing time of *Bti* produced with various quantities of chicken feather hydrolysate mixed with delignified rice straw (NaOH) with additional Yeast supplementation. Biomass production with various concentrations of CFH (0.1, 0.2, 0.3, 0.4, 0.5) were 0.179, 0.189, 0.193, 0.187, 0.177 g. 0.3ml was found be optimum, with a sporulation time of 39-45 hours and killing time of 214 minutes. The biomass produced is lower compared to similar condition with fish hydrolysate, since fish proteins (Globular proteins) are more digestible or easily

hydrolysable than poultry feather, which is beta pleated sheet structure.

4.6.2. Batch fermentations of *Bti* using delignified rice straw with CaOH, supplemented with chicken feather hydrolysate

Table 7 shows the biomass, sporulation time and larvicidal assay killing time with various quantities of chicken feather hydrolysate mixed with delignified rice straw (CaOH) with additional yeast supplementation. Biomass production with various concentrations of CFH (0.1, 0.20.3, 0.4, 0.5) were 0.176, 0.183, 0.213, 0.211, 0.1191 g. 0.3ml was found be optimum, with a sporulation time of 39-43 hours and killing time of 234 minutes. The biomass produced is lower (as in previous case with NaOH) compared to similar condition with fish hydrolysate, since fish proteins (Globular proteins) are easily hydrolysable than poultry feather.

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