Bioprocessing of Agricultural waste (Banana pseudostem) for Cellulase Production by Solid-State Fermentation

Abstract – *Cellulase was produced by Cellulomonas uda utilizing banana stem waste in solid state fermentation (SSF). The effect of varying particle size, extraction pH, incubation pH, incubation temperature, activity temperature, incubation period, moisture content, peptone and yeast extract content on the production of cellulase was investigated. The maximum activity of cellulase produced by Cellulomonas uda on banana waste for particle size(6.97 IU/min for 1mm), extraction pH(7.13 IU/min for pH 7), incubation pH(6.97 IU/min for pH 7), extraction temperature(7.10 IU/min for 50^0C), incubation temperature(7.20 IU/min for 45^0C), incubation period(7.20 IU/min on 3rd day), moisture content(7.12 IU/min for 100%), peptone content(7.23 IU/min for 0.5 gm) and yeast extract content(7.18 IU/min for 0.30gm) was recorded. The enzyme produced by Cellulomonas uda can be used in industrial processes after characterization.*

Key words: Banana Waste, cellulase, *Cellulomonas uda*, Solid state fermentation.

[I] INTRODUCTION

Cellulose is the most common organic polymer, representing about 1.5×10^{12} tons of the total annual biomass production through photosynthesis especially in the tropics, and is considered to be an almost inexhaustible source of raw material for different products [1,2,3]. It is the most abundant and renewable biopolymer on earth and the dominating waste material from agriculture [4]. A promising strategy for efficient utilization of this renewable resource is the microbial hydrolysis of lignocellulosic waste and fermentation of the resultant reducing sugars for production of desired metabolites or biofuel. Microbial degradation of lignocellulosic waste and the downstream products resulting from it is accomplished by a concerted action of several enzymes, the most prominent of which are the cellulases, which are produced by a number of microorganisms and comprise several different enzyme classifications. Media used in cellulose fermentation contain cellulose in different degree of purity [5] or as raw lignocellulosic substrate [6], which is true in case of solid state fermentation. Cellulases hydrolyze cellulose (ß-1,4-D-glucan linkages) and produce as primary products glucose, cellobiose and cello- oligosaccharides. There are three major types of cellulase enzymes [Cellobiohydrolase (CBH or 1,4- ß-D-glucan cellobiohydrolase, EC 3.2.1.91), Endo-ß-1,4-glucanase (EG or endo-1,4-ß-D-glucan 4- glucanohydrolase, EC 3.2.14) and ß-glucosidase (BG-EC 3.2.1.21)] [7]. Cellulases have became the third largest group of enzymes used in industry application [8] other then lipase [9,10,11,12,13], xylanase [14,15,16], amylase [17], protease[18] and pectinase [19]. Cellulases are used in the textile industry [20], in detergents [21,22], pulp and paper industry [23], improving digestibility of animal feeds [24] and in food industry [25]. In food industry, cellulases are used in extraction and clarification of fruit and vegetable juices, production of fruit nectars and purees, and in extraction of olive oil [25], Glucanases are added to improve the malting of barley in beer manufacturing [26] and in wine

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industry, better maceration and color extraction is achieved by use of exogenous hemicellulases and glucanases [25]. In the pulp and paper industry, cellulases and hemicellulases have been employed for biomechanical pulp and hand sheet strength properties [27], deinking of recycled fibers [28]. Extracellular enzymes are also used in synthesis of nanoparticles [29, 30, 31, 32,33].

A potential application of cellulose is the conversion of cellulosic materials to glucose and other fermentable sugars, which in turn can be used as microbial substrates for the production of single cell proteins or a variety of fermentation products like ethanol [34,35].Commercial production of cellulases has been tried by either solid or submerged culture including batch, fed batch, and continuous flow processes [1, 2, 3].Various statistical tools or methods are used in order to optimize production of cellulase or biomolecules by using fermentation process [36,37,38,39,40].

The main agricultural waste of Jalgaon district is the banana fruit stalk and pseudo stem, also accumulated as waste, posing serious environmental problems. Banana fruit stalk contains 56.8% total sugar, 27.0% starch, 4.65% reducing sugar and 4.3% protein on a dry weight basis. The Banana pseudo stem consists of Hemicellulose 65%, Cellulose 64%, Lignin 19%, and Ash 15% on dry weight basis. The microbial strains can be used to utilize the banana waste, food processing waste and domestic waste for production of enzymes and organic solvents/acids.

[II] MATERIALS AND METHODS

2.1.Collection of substrate.

The main agricultural waste of Jalgaon District (called land of Banana) is banana pseudostem. Pseudostem rich in cellulose accumulate as waste in the banana field. This pseudostem collected from the banana fields around Jalgaon city is utilized as substrate in SSF. Banana pseudostem collected from the fields near North Maharashtra University, Jalgaon.

2.2.Microorganism

Microorganism was obtained from National Center for Industrial Microorganism (NCIM), a division at National Chemical Laboratory (NCL), Pune. *Cellulomonas uda* with NCIM No. 2353 (Cellulase producer) is maintained on nutrient agar slant (Peptone 5 gm, Beef extract 3 gm, NaCl 5 gm, Distill water 1000 ml) of pH 7 at 4^0C..The microorganism is subcultured at regular interval in the department laboratory.

2.3.Preparation of Substrate

Banana stem used as substrate for the production of cellulase enzyme was obtained from farm land located back side of North Maharashtra University, Jalgaon. The collected substrate was chopped in to smaller pieces and sun dried for 48 hrs. The sun dried substrate was collected and then oven dried at 70 ^{0} C for 24 hrs. The substrate was then ground to powder in an electric grinder.

The above powdered substrates were passed through sieve shaker of 2mm mesh size. Substrate after sieve analysis were collected in 250 ml conical flask or petriplates and moistened with salt solution containing gm / 100 ml : yeast extract 0.3, peptone 0.5, NaCl 1.5,$Na_2HPO_4.2H_2O$ 1.1, NaH_2PO_4 0.61, KCl 0.3 and $MgSO_4.7H_2O$ 0.01 in laminar air flow [3, 17]. The substrates were moistened to 150% (W/V) by salt solution. Moistened substrate was taken in to autoclave and sterilized for 15 minute at 121 ^{0} C for proper cooking of the substrate and to increase its amenability for microorganisms.

2.4.Inoculum Preparation

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cellulomonas uda (NCIM No. 2353, Cellulase producer) cells were transferred aseptically to 100 ml conical flask containing 50 ml of sterilized inoculum medium (sterilized at 121^0C for 15 minutes) containing g/100ml: glucose 2, yeast extract 0.3, peptone 0.5, NaCl 1.5,Na2HPO4.2H2O 1.1, NaH2PO4 0.61, KCl 0.3 and MgSO4.7H2O 0.01 in laminar air flow. The flask was then kept in incubator at 37 ^{o}C for 48 hrs. The homogenous cell suspension (10^6 – 10^7 cells / ml) was used as inoculum.

2.5.Solid State fermentation

After sterilizing, the substrate was cooled to room temperature. Substrate of 10 gm in petriplate and 15 gm in conical flask of 250 ml was added with the inoculum of 10 % (W/V) in the laminar air flow with the help of sterilized pipette.

Cellulomonas uda (NCIM No. 2353, Cellulase producer) was inoculated on banana stem After inoculation the flask and petriplates were incubated at 37 ^{0} C for 3 days. The SSF media flasks and petriplates were gently shaken after every 12 hrs for uniform mixing of the substrate and microorganism.

2.6.Enzyme Extraction

After incubation, the fermented banana stem waste sample was extracted with 1:10 (W/V) of 0.1 M sodium phosphate buffer of pH 6.9 for 60 minutes at 150 rpm. The material was filtered through muslin cloth. Filtrate collected was centrifuged at 10000 rpm for 10 minutes at room temperature. Supernatant was carefully collected and was used as crude enzyme extract for determining cellulase activity [17].

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2.7.Enzyme Assay

Cellulase enzyme activity was estimated by filter paper method. Cellulase acts on cellulose to produce reducing sugar (glucose) and measured by DNSA method. Chemicals used for enzyme estimation were 0.1 M sodium phosphate buffer of pH 6.9, filter paper discs, DNSA, potassium sodium tartarate, glucose solution. 1 ml of crude enzyme extract was added to 30 mg of dry whatmann filter paper 1 and incubated the mixture for 1 hr at 50^0 C. DNSA reagent of 2.5 ml was added and mixture was heated in a boiling water bath for 15 minutes. Absorbance was measured at 530 nm. Standard graph of glucose was prepared. The enzyme activity was expressed as µmol of glucose released per minute.

2.8.Parameters

The effect of varying particle size, extraction pH, incubation pH, incubation temperature, activity temperature, incubation period, moisture content, peptone and yeast extract content on the production of α-amylase was investigated.

[III] RESULTS AND DISCUSSION

3.1. Effect of particle size on Enzyme activity

Effect of particle size on enzyme activity (cellulase) was studied by taking banana stem waste as substrate. After grinding the substrate the substrate of different particle size from 0.100 mm to 2 mm was taken to study the effect of particle size on enzyme activity. Sieve shaker was used to separate the substrate particle of different size. Sieves of different mesh size arranged in a decreasing order of mesh size as 2 mm , 1.4 mm ,1 mm , 0.850 mm, 0.425 mm, 212mm, 106 mm were mounted on a vibrator. Substrate of different particle size was considered based on under size of particle size. Substrate of each particle size was taken in conical flask (250 ml) and solid state fermentation was carried out for 48 hrs. at 37 ^{0} C. The crude enzyme was extracted; a reading of each particle size was recorded for enzyme activity. The mean reading of

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enzyme activity against particle size is shown in Fig No1. Larger particles provide better respiration/aeration efficiency due to increase of interparticle space. In contrast, a small substrate particle may result in substrate accumulation, which may interfere with microbial respiration/aeration and therefore result in poor growth and enzyme production. For cellulase activity, decrease in particle size from 2mm to 1mm shows increase in enzyme activity, but further decrease in particle up to 0.106mm decreases enzyme activity. Optimal activity of 6.97 IU/min was seen in 1mm particle size.

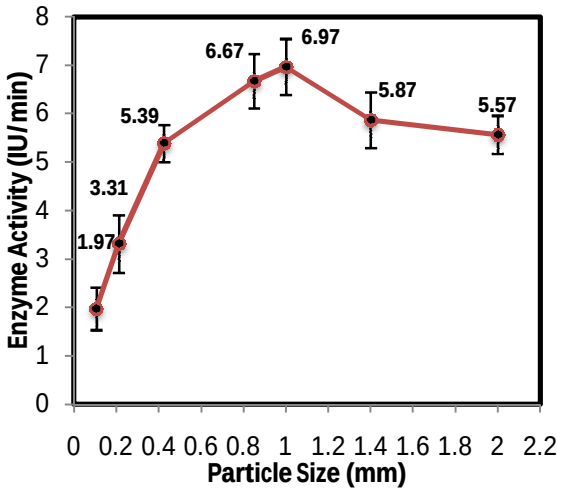

Fig No: 1 Effect of particle size on enzyme activity

To investigate the effect of extraction pH, incubation pH, incubation temperature, extraction temperature, incubation period, moisture content, peptone and yeast extract content on enzyme activity, 1mm particle size was used for the solid state fermentation.

3.2.Effect of extraction pH on enzyme activity

Solid state fermentation was performed to check the effect of extraction pH of enzyme activity. Crude enzyme was extracted by using buffers of different pH from 4 to 9. Acetate buffer of 0.2M was used for pH 4 and 5, Phosphate buffer of 0.1M was used for pH 6, 7, 8 and Glycine buffer of 0.2M was used for pH 9.The enzyme activity was recorded to study the effect of pH of extracting buffer and also to optimize the condition for pH. Increase in the hydrogen ion concentration considerably influences the enzyme activity. Each enzyme has an optimum pH at which the activity is maximum. Hydrogen ions influences the enzyme activity by altering the ionic charges on the amino acids particularly at the active site, substrate etc. for cellulase activity, increase in pH from 4 to 7 increases enzyme activity, further increase in pH up to 9 decreases enzyme activity. Optimal activity of 7.13 IU/min was observed at pH 7 as shown in Fig No.2. Muhammad Irfan *et al*. 2010 reported production of carboxymethyl cellulase by thermophilic *Trichoderma viride* on wheat straw and got optimum activity for extraction pH at pH 5.5 [41].

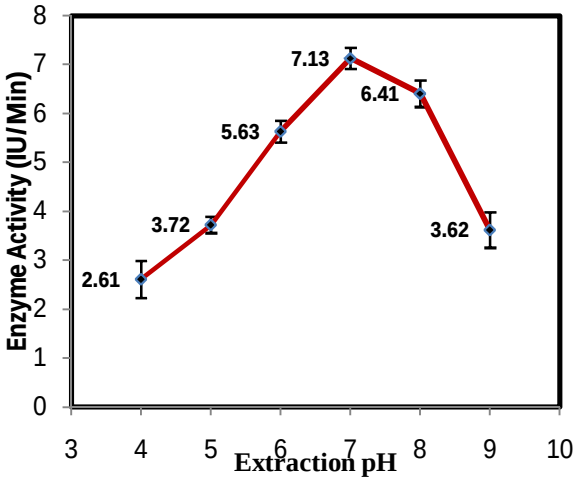

Fig No:2 Effect of extraction pH on enzyme activity

3.3.Effect of initial pH on enzyme activity

Solid state fermentation was performed to study the effect of incubation pH on enzyme activity. Among the physicochemical parameters, pH of the growth medium plays an important role by inducing morphological changes in the organism and in enzyme

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secretion. Variation of pH results due to substrate consumption (eg: protein hydrolysis) and metabolite production like organic acids. For cellulase activity, increase in pH from 4 to 7 increases enzyme activity, further increase in pH up to 9 decreases activity. Optimal activity of 6.97 IU/min was observed at pH 7. Muhammad Irfan *et al*. 2010 reported production of carboxymethyl cellulase by thermophilic *Trichoderma viride* FBL1 on wheat straw and got optimum activity at pH 5 [41]. S. Shafique *et al*. 2004 reported production of exoglucanase by *Bacillus subtilis* on banana stalk and got optimum activity at pH 7 [42]. Ikram Ul Haq *et al*. 2006 reported production of cellulase by *Trichodrma harzianum* on agricultral by products and got optimum activity at pH 6.5 [43]. Ishtiaq Ahmed *et al*. reported production of cellulase by *Trichoderma viride* on wheat straw and got optimum activity at pH 5.5 [44].

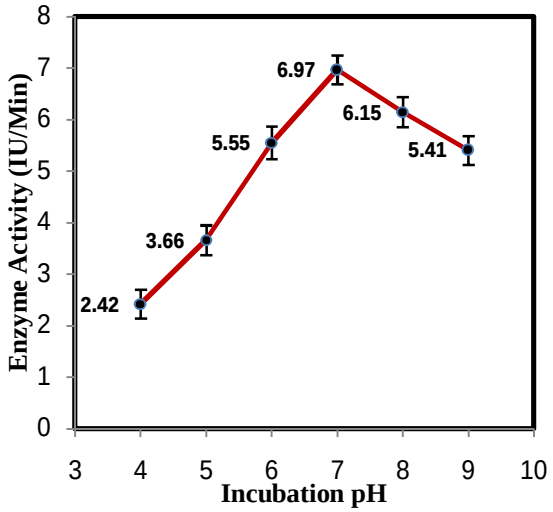

Fig No:3 Effect of incubation pH on enzyme activity

3.4.Effect of incubation temperature on enzyme activity

Solid state fermentation was performed to study the effect of incubation temperature on enzyme activity. Incubation temperatures used were 20^0 C, 25^0 C, 30^0C, 35^0C, 40^0C, 45^0C and 50^0C. After inoculation of substrate, it was kept in incubator at different temperature for 3 days. Crude enzyme was extracted and activity was measured as shown in Fig No. 4. Bacterial cells have various mechanisms that allow them strictly to control enzyme excretion. Change in the nature of cell envelope can affect the release of extracellular enzymes to the culture medium. Temperature is one of the factors that induce such changes on cell membrane and cell wall. for cellulase activity, increase in temperature from 25^0C to 45^0C, increases enzyme activity, whereas increase in temperature to 50^0 C decreased the enzyme activity. The optimum enzyme activity obtained was 7.20 IU/min at 45^0C. Muhammad Irfan *et al*. 2010 reported production of carboxymethyl cellulase by thermophilic *Trichoderma viride* on wheat straw and got optimum activity at 40^0C [41]. Shafique *et al*. 2004 reported production of exoglucanase by *Bacillus subtilis* on banana stalk and got optimum activity at 35^0C [42].Ikram Ul Haq *et al*. 2006 reported production of cellulase by *Trichodrma harzianum* on agricultral by products and got optimum activity at 28^0C [43].Ishtiaq Ahmed *et al*. reported production of cellulase by *Trichoderma viride* on wheat straw and got optimum activity at 40^0C [44].

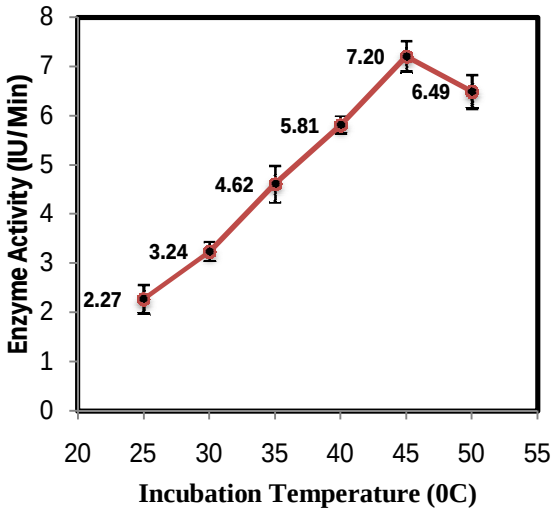

Fig No:4 Effect of incubation temperature on enzyme activity

3.5.Effect of extraction temperature on enzyme activity

Solid state fermentation was performed to study the effect of extraction temperature on enzyme activity. Temperatures used were 25^0 C, 30^0C, 35^0C, 40^0C, 45^0C and 50^0C. After extraction of enzyme, activity was measured at different temperature. Enzyme activity increases with increase in temperature to a maximum and then declines. Increase in temperature results in higher activation energy of the molecules and more molecular collision and interaction of the reaction to proceed faster. When enzymes are exposed to a temperature above maximum denaturation leads to dearrangement in the native structure of the protein and active site, which results in inactivation of enzymes. for cellulase activity, increase in temperature from 25^0C to 50^0C increases enzyme activity. The optimum enzyme activity obtained was 7.10 IU/min at 50^0C as shown in Fig No. 5. Muhammad Irfan *et al*. 2010 reported production of carboxymethyl cellulase by thermophilic *Trichoderma viride* on wheat straw and got optimum activity at 55^0C [41].

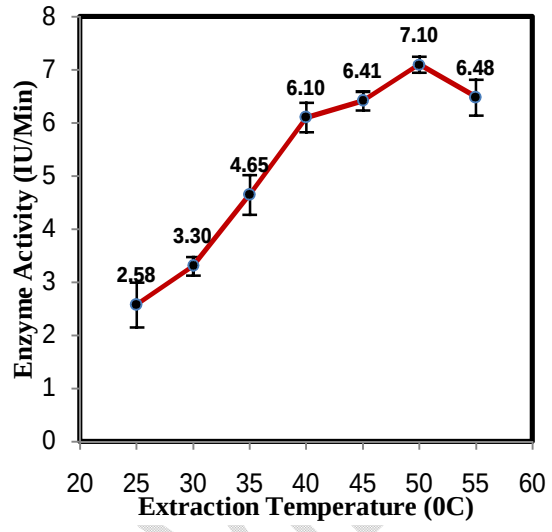

Fig No: 5 Effect of extraction temperature on enzyme activity

3.6.Effect of incubation period on enzyme activity

Solid state fermentation was performed by varying incubation period from 1 day to 10 days at 45^0C. For cellulase activity, increase in incubation period from 1 day to 3 days increases enzyme activity, whereas increase in incubation period from 3 days to 10 days showed decrease in enzyme activity. Optimum activity of 7.20 IU/min was observed at 3rd day of incubation due to availability of desired moisture in the substrate, whereas drastic decrease in enzyme activity was observed after 3rd day due to decrease in moisture content of the substrate. Muhammad Irfan *et al*. 2010 reported production of carboxymethyl cellulase by thermophilic *Trichoderma viride* on wheat straw and got optimum activity for incubation period at 7th day incubation.[41]. MAM Abo-State *et al* 2010 reported production of cellulase by *Aspergillus terreus* mam-F23 and *Aspergillus flavus* mam-F35 on wheat straw and got optimum activity for incubation period at 48 hrs and 60 hrs respectively [45]. S. Shafique *et al*. 2004 reported production of exoglucanase by *Bacillus subtilis* on

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banana stalk and got optimum activity at 72 hrs of incubation [42]. Ezyana Kamal Bahrin *et al*. 2011 reported production of cellulase by *Botryosphaeria sp.* from oil palm empty fruit bunch and got optimum activity at 3rd day of incubation [46]. C. Pothiraj et al, 2006 reported production of cellulases by various fungal cultures like *Rhizopus stonifer, Aspergillus niger and Aspergillus terreus* on cassava waste and got optimum activity at 10 days, 8 days and 8 days of incubation period respectively [47]. Ikram Ul Haq *et al*. 2006 reported production of cellulase by *Trichodrma harzianum* on agricultral by products and got optimum activity at 72 hrs of incubation [43].

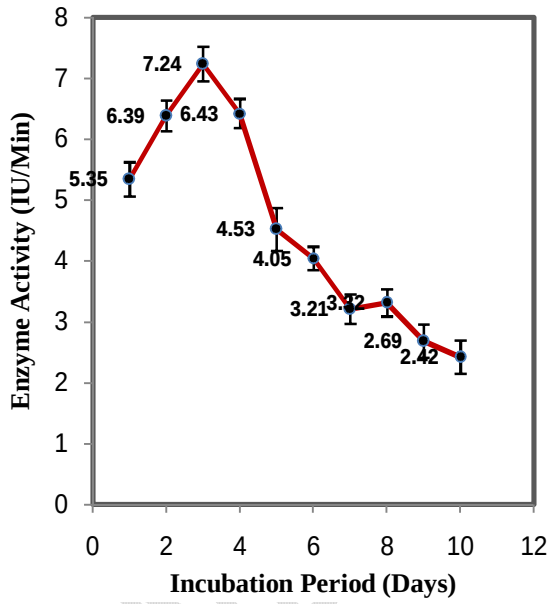

Fig No: 6 Effect of incubation period on enzyme activity

3.7.Effect of initial moisture content on enzyme activity

Grinded substrate of 1mm particle size was taken to study effect of moisture content on enzyme activity. Substrate was cooked with salt solution by adding salt solution (w/v) to get different moisture content (50 to 120%). Initial moisture contents of the substrate are known to critically influence microorganism growth and enzyme production in solid state fermentation. Presence of water in the substrate makes the nutrients more easily accessible for microorganism growth. Moreover, water has an impact on physicochemical properties of the substrate, which in turn affect the enzyme production. Higher water causes reduction in enzyme yield due to steric hindrance of the growth of the producer strain by reducing porosity of the solid matrix, causes particles to stick together thus interfering adversely the oxygen diffusion in the substrate. Lower moisture content causes reduction in solubility of nutrients of the substrate, low degree of swelling and high water tension. for cellulase activity, increase in moisture content from 50% to 100% increases enzyme activity, further increase in moisture content of substrate from 100% to 120% decreases enzyme activity. Optimum enzyme activity of 7.12 IU/min was observed at 100% moisture content of the substrate as shown in Fig No.7. Muhammad Irfan *et al*. 2010 reported production of carboxymethyl cellulase by thermophilic *Trichoderma viride* on wheat straw and got optimum activity for initial moisture content at 40% [41]. S. Shafique *et al*. 2004 reported production of exoglucanase by *Bacillus subtilis* on banana stalk and got optimum activity at 70% of initial moisture content [42]. Ishtiaq Ahmed *et al*. reported production of cellulase by *Trichoderma viride* on wheat straw and got optimum activity at 40% of initial moisture content [44].

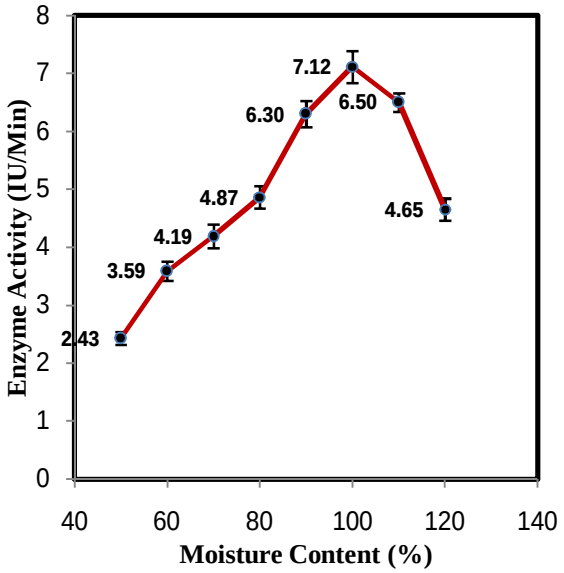

Fig No: 7 Effect of initial moisture content on enzyme activity.

3.8.Effect of peptone and yeast extract content on enzyme activity

Productions of hydrolytic enzymes are enhanced by the additional nitrogen source like peptone and yeast extract. In this work effect of different concentrations of peptone and yeast extract was checked on enzyme activity. for cellulase activity, increase in peptone content from 0.1 gm to 0.5 gm and yeast extract content from 0.06 to 0.30 gm increases enzyme activity, further increase in peptone content from 0.5 to 1.0gm and yeast extract to 0.48gm decreases enzyme activity. Optimum activity of 7.23 IU/min was observed at 0.5gm of peptone content and 7.18 IU/min at 0.30gm of yeast extract as shown in Fig No.8 and 9 respectively.

S. Shafique *et al*. 2004 reported production of exoglucanase by *Bacillus subtilis* on banana stalk and got optimum activity at 0.1% (w/w) of peptone and 0.4% (w/w) of yeast extract content [42].

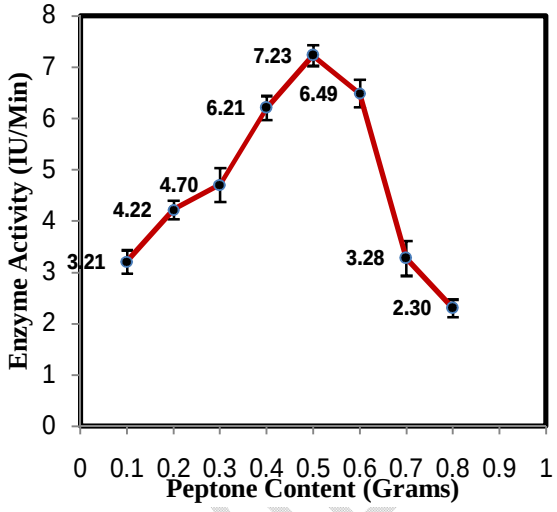

Fig No: 8 Effect of peptone content on enzyme activity

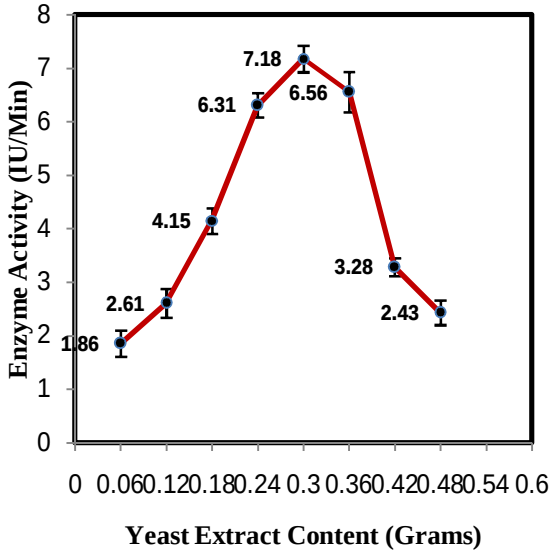

Fig No: 9 Effect of yeast extract content on enzyme activity

[IV] CONCLUSION

SSF offers numerous advantages over submerged fermentation; these include high productivity, relatively higher concentration of products, less effluent generation and simple fermentation equipment. Banana stem waste provides a low cost feed stock for biological production of cellulase. In present study, cellulase produced by *Cellulomonas uda* (NCIM No.2353) was

obtained from NCIM, NCL, Pune. The maximum activity of cellulase produced by Cellulomonas uda on banana waste for particle size(6.97 IU/min for 1mm), extraction pH(7.13 IU/min for pH 7), incubation pH(6.97 IU/min for pH 7), extraction temperature(7.10 IU/min for 50^0C), incubation temperature(7.20 IU/min for 45^0C), incubation period(7.20 IU/min on 3rd day), moisture content(7.12 IU/min for 100%), peptone content(7.23 IU/min for 0.5 gm) and yeast extract content(7.18 IU/min for 0.30gm) was recorded. Thus banana stem waste could be a potential, economic source for the production of cellulase by solid state fermentation.

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