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Economical Cellulase Production under Optimized condition in Batch condition using Water Hyacinth (WH) Waste

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Abstract

Currently research attempted for enhanced cellulase production using Water hyacinth waste. We found that . Trichoderma reesei selected with WH for cellulase production. Optimization the effective conditions for production of CMCase temp-40 ºC, pH-5, tween80 3%, WH7.5%, nitrogen source 1% as peptone, incubation time 7days, inoculum 5% at rotation 100 rpm for FPase production only two condition was difference inoculum was higher (10%) and rotation speed was 150 rpm. Production of cellulase for CMCase was 52% more increase activity observed after media optimization and similar for FPase was 84% increase activity observed after media optimization.

Keywords: *CMCase, FPase, Cellulase, optimization, DOE, Trichoderma reesei selected, Biofuel*

1.1 Introduction:

Constant depletion of fossil fuel is of great concern. Therefore only alternative is large scale production of biofuel to meet its demand for blending purpose. High Cellulose available from various agricultural waste around the Globe (around 10^{12} tons of the total annual biomass) may be an ideal substrate for its conversion into bioethanol (Bhatia and Johri 2015). Unfortunately, conversion of crystalline cellulose into amorphous one is a major hurdle due to presence of recalcitrant, which requires judicial selection of pretreatment techniques. Cellulase attacks in stepwise manner for degradation of cellulose first into disaccharides (cellobiose) which is further degraded by beta glucosidase (cellobiases) into monomeric form.

Currently research attempted for enhanced cellulase production using Water hyacinth waste

Reviews of literature

Pre-treatment is one of the important criteria in reducing the lignin content present in lignocellulosic biomass (Bhatt & Shilpa, 2015; Jung, Kim, Kim, & Kim, 2013). Chemical pretreatment have been done by various workers like inorganic chemicals such as HCl and NaOH and organic chemicals such as lactic acid, citric acid e.t.c. (Anita Singh and Bishnoi 2013). The feature of an effective pre-treatment is, low degradation of hemicellulose, low inhibitor formation after cellulose hydrolysis, low energy requirements and cost-effective (Martinez et al. 2001). There are three types of preferred pre-treatment strategies such as 1- physical, 2-chemical, 3-physicochemical and biological pre-treatment. Most often physical pre-treatment such as breaking of larger cellulose particle is essential before application of other methods such as chemical or biological. Some chemical methods such as Alkaline pre-treatment is preferred over acid pre-treatment on the basis of its capability to dissolve lignin rapidly at room temperature, with less degradation of hemicellulose (Kumar, Singh, and Ghosh 2009) and thus high recovery of glucose yield (Nigam 2002).

In one report cellulase production have been done by using waste WH from Rhizopus oryzae MTCC 9642 in submerged and solid state fermentation and media condition were optimized. In optimised condition FPase activity reported in submerged fermentation was substrate concentration (w/v) 1.25%, pH 7.32, and temperature 25 ºC while in SSF mode the substrate concentration. 0.5%, pH 6.0 and temperature 18 ºC (Karmakar and Ray 2011).

In an another work Deshpande et al., 2009 optimised the cellulase production by Trichoderma reesei from water hyacinth. In the optimised condition cellulase activity reported

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was 0.22 ± 0.04 IU/ml (approximately 73.3 IU/g cellulose) after 15-days with specific activity of 6.25 IU/mg protein. Saccharification rate of this enzyme was around 28.7% (1% water hyacinth) with 1.2 IU/g cellulase enzyme. With mixture of microbes such as T. reesei QM 9414 mutant and P. chrysosporium, high cellulase production was reported with WH as compared to wheat bran in SSF mode where liquid/solid ratio was 2.5 with 10 days incubations. However beta glucosidase ceased using WH as a substrate.(Deshpande, Nair, and Khedkar 2009). Cellulase production has been reported by various cellulolytic microbes as briefed in Table 2.2. One of the extensively used fungi for cellulase production is T. reesei. Tangnu,1981 reported production of both cellulases and hemicellulase using Trichoderma reesei RutC-30in different cellulose concentrations (1, 2.5, and 5.0%) in submerged conditions (Tangnu, Blanch, and Wilke 1981). Devi and Kumar (2012) isolated celluloytic microbes from local industrial wastes like paper, timber and saw mills e.t.c which resulted in screening of Trichoderma reesei from paper waste. The maximum enzyme was produced was 3.9 IU at 45°C and pH 5 after 7th day. (Devi and Kumar 2012,).

Table 1 Cellulase producing fungal strain

Fungal strain	Reference
P.chrysogrnum	(Chinedu et al. 2007)
A.heteromorphus	(Ajay Singh, Kuhad, and Ward 2007)
Botsysatra	(Shoemaker and Brown 1978)
A.fumigotus	(Hamilton and John Wase 1991)
Chaetomium sp.NIOCC36	(Ravindran, Naveenan, and Varatharajan 2010)
Fomitopiss sp. RCK 2010	(Deswal, Khasa, and Kuhad 2011)
Scopulariopsis	(Kodali & Pogaku, 2006)
Neuraspora	(Sharada, Venkateswarlu, Venkateshwar, & Rao, 2013)
Tricothecium roseum	(Shanmugam, Mani, & Narayanasamy, 2008)
A.candidus	Milala et al.,2009 Milala,M.A., Shugaba,A., Gidado,A., Ene,A.C. and Wafae,J.A., 2005. Studies on the use of agricultural wastes for cellulase enzyme production by Aspergillus niger. Res. J. Agr. And Bio. Sci., 1(4): 325-328.
A.japonicus	Sharma et al., 1985 Sharma,A., Milstein,O., Vered,Y., Gressed,J. and Flowers,B.M., 1985. Effect of aromatic compounds on hemicelluloses degrading enzymes in Aspergillus japonicas. Biotechnol. Bioeng., 27: 1095-1101.
T.aureoviride	Mercedes et al.,2001 93. Mercedes Zaldivar, Juan Carlos Velasquez, Ines Contreras, Luz Maria Perez., 2001. Trichoderma aureoviride 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and / or biocontrol. Electronic Journal of Biotechnology, 4(3): 0717-3458.
Pesalotia	Metha et al.,1975 Metha,P., Vyas,K.M. and Sakeena,S.B., 1975. Effect of native carbon sources and pH on the cellulases of Alternaria solani and Aspergillus terreus. Science and culture, 41: 401.
Stachy	Mandels et al.,1976 Mandels,M. and Sternberg,D., 1976. Recent advances in cellulases technology. J. Ferment. Technol., 54(4): 267-286.
Polmarum	Kassim et al., 1983 Kassim,E.A., 1983. Cellulase enzyme from Aspergillus niger. J. Fac. Sci. Riyadh Univ., 16.
Trichoderma reesei	Muthuvelayudham, et al.,2006 Muthuvelayudham, R. and Viruthagiri, T., 2006. Fermentative production and kinetics of cellulase protein on Trichoderma reesei using sugarcane bagasse and rice straw. Afr. J. Biotechnol., 5 (20): 1873-1881.
A.niger	Gokhle et al.,1984 Gokhale,D.V., Puntamberkar,U.S., Vyas,A.K., Patil,S.G. and Deobagkar,D.N., 1984. Hyper production of β-glucosidase by an Aspergillus species. Biotechnol. Lett., 6: 719-722.
Tricothecium reseum	Prashath et al., 2008 Prashanth Shanmugam, Madhumathi Mani and Mathivanan Narayanasamy., 2008. Biosynthesis of cellulolytic enzymes by Tricothecium roseum with citric acid mediated induction. Afr. J. Biotechnol., 7(21): 3917-3921.
Merulius	Takao et al.,1985 Takao,S., Kmagata,Y. and Sasaki,H., 1985. Cellulase production by Penicillium purpurogenum. J. Ferment. Technol., 63(2): 127-
Aoryzar	Adeleke et al.,2012 Adikanae,H.V.and Patil,M.B., 1983. Cellulase production by Fusarium solani. Indian Bot. Rep., 2(1): 97-98.

2. Methodology

Culture collection

T. reesei (MTCC No.164) cultures were procured from IMTECH, Chandigarh. As per directions, the T.reesei culture was revived on Potato Dextrose Agar (PDA) at 23ºC.

Sample collection and pretreatment

Water hyacinth leaves were collected from the local pond in Phagwara and washed properly with distilled water and then sun dried leaves crushed to make a powder.

a) Steam explosion (Physical treatment)

The WH powder was autoclaved for different time intervals i.e. for 20 and 30 minutes using distilled water at 121°C at 15 psi and pressure was released by sudden opening of valve.

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B) Chemical pre-treatment

(I) Sodium Hydroxide Pre-treatment

Different concentrations (1, 2, 4, 6, 8 and 10%) of Sodium hydroxide was used with 10 grams of WH powder and allowed to stand at 37°C for 4 hr and was autoclaved at 121°C for 30 min with sudden release of pressure. The solution was washed and filtered to collect the powder with tap water until neutral pH was reached, again filtered and dried at 65°C.

(II) HCl Pre-treatment

Different concentrations of HCl were used in the range 1, 2, 4, 6, 8 and 10 % with 10 grams of WH powder at 37°C for 4 hrs and then autoclaved at 121°C, 15psi for 30 mins. The powder was collected washed extensively with tap water until neutral pH was reached, and sample were filtered and dried at 65°C.

(III) Lactic acid Pre-treatment

Different concentrations of Lactic acid were used in the range 1, 2, 4, 6, 8 and 10 % with 10 grams of WH powder at 37°C for 4 hrs and then autoclaved at 121°C, 15psi for 30 mins. The powder was collected and washed extensively with tap water until neutral pH was reached, and then filtered and dried at 65°C.

SSF fermentation

The fungal spores of T. reesei grown on PDA plate for 6 days were inoculated in the production media as described by Mandel (1957). The 10% w/v of pre-treated WH powder was added to 250 ml Erlenmeyer flasks with fungal culture T. reesei.

Optimization of factors for Cellulase production

Taguchi statistical methodology has been applied for Design of experiments with selected eight variables viz. effect of temperature, pH, time of incubation, inoculum level, Nitrogen source, WH concentration, Tween-80, and revolution per minutes on cellulase production using the software Qualitek 4. The M18 array were fit for selected variable and 18 experimental design were obtained by Qualitek-4 software (Nutek Inc., MI).

In brief, we can describe Taguchi Optimization methodology in five simple steps.

1- Selection of factors and their concentrations level (variables)

2- Selection of proper orthogonal array.

3- Setting of experiments as per design of experiments and getting results in three trials.

4- Statistical analysis ANOVA for determination of the main influencing factor.

5- Prediction of optimum condition and validation of results by actual experiment.

Determination of significant factors: *The factors showing difference in levels shows significant influence on cellulase production were determined based on level difference. In addition the interaction between two factors gives a better insight into the overall process analysis. Any individual factor may interact with any or all of the others factors creating the possibility of presence of a large of interactions. This kind of interaction is possible only in Taguchi DOE methodology. Analysis of experimental data and prediction of performance phase 3. The result obtained were analysed was based on the S/N ratio ANOVA. The result obtained after the data processing by Qualitek-4 software.*

Analysis of sample

Sample were analysed from supernatant obtained after centrifugation of hydrolysate at 2000 rpm for 15-20 min at 4°C and the cell free culture supernatant fluid was further used for cellulase assay.

a) FTIR analysis

2 mg of untreated and pre-treated solid mass of WH were used for FTIR analysis (Lovely Professional University). WH samples were prepared by mixing with 200mg of spectroscopic grade KBr. Untreated WH powder was used as a standard for FTIR analysis against treated samples.

b) Anthrone Assay (Cellulose estimation)

Cellulase estimation was carried out as per protocol of Updegraff (1969).

c) Enzyme Assay

CMCase and FPase activities *assayed were by the method reported by Ghose 1987 using CMC as substrate and Whatman No.1 filter paper as substrate respectively. According to International Union of Biochemistry "One international unit of enzyme (1IU) corresponds to the amount of enzyme required to release 1 micromole of reducing sugars per minute during the hydrolysis reaction"*

I) CMCase assay

For CMCase assay 1 ml of diluted enzyme solution was added to 1% carboxymethyl cellulose solution The reaction mixture was incubated for 30 minutes at 50°C and 3 ml of DNS reagent was added .The test tubes were boiled for 15 minutes and 1ml of 40% sodium potassium tartrate was added. The test tubes were cooled and absorbance was measured at 550 nm.

II) Estimation of reducing sugars

Reducing sugars concentration was estimated by a method reported by Miller 1959.

Results and Discussion

Pre-treatment studies

The Dried WH powder was treated with 8% of NaOH, HCl and Lactic acid. Water hyacinth powder after pre-treatment process shown in figure 1.(a & b)

Fig 1: (a) Water hyacinth (leaves powder) (b) Water Hyacinth powder treated with different conc. of NaOH

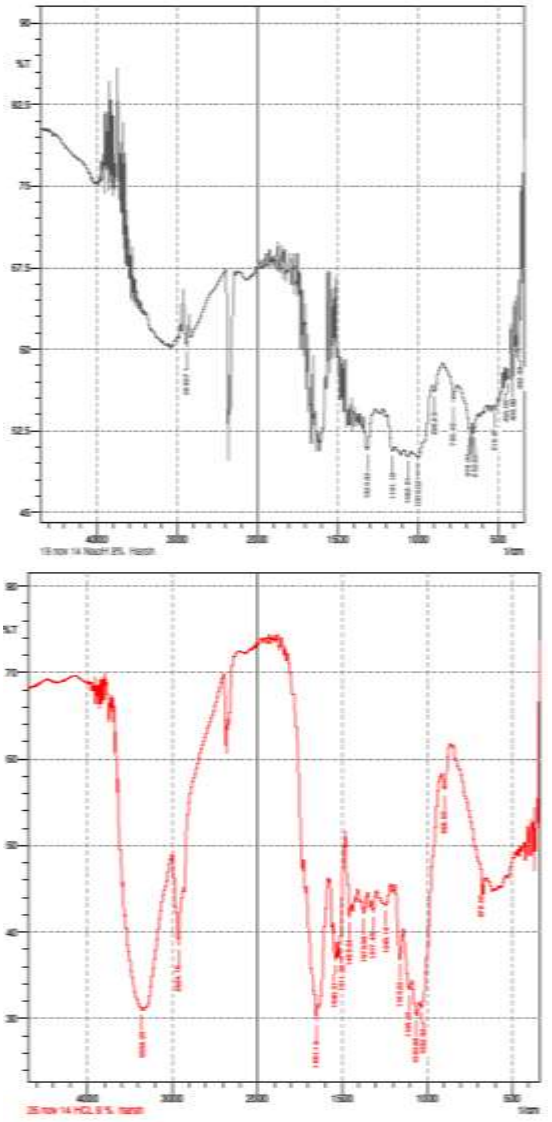

Fig. 2 (a) 8% NaOH
Fig. 2 (b) 8% HCl

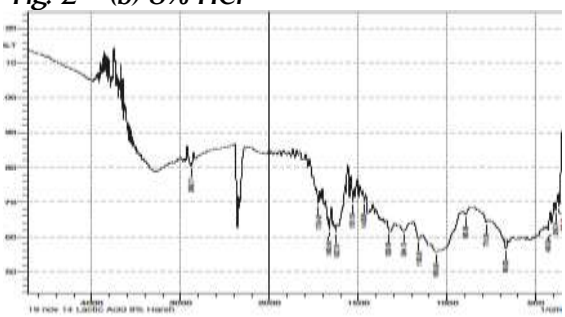

Fig. 2 (C) 8% Lactic acid

6.1 Optimization of parameters for Enzyme production

The parameters selected in this work were temperature, pH, Tween 80, Water hyacinth, Nitrogen source, Time of incubation, Inoculum level and Rotations was shown in table 3. On the basis of selected factor L18 design were obtained. Shown in Table 3 and table 4.

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Table 2: Factors selected for optimization of media.

Factors	Level 1	Level 2	Level 3
1. pH	*3*	*5*	*7*
2. Temperature (°C)	*30*	*40*	
3. Nitrogen source	*Yeast extract*	*Peptone*	*Beef extract*
4.WH (%)w/v	*7.5*	*10*	*15*
5. Tween 80 (%) v/v	*0.5*	*1.5*	*3*
6. Inoculum conc. (%)	*5*	*7*	*10*
7. Rotation (rpm)	*0*	*100*	*150*
8. Incubation time(days)	*3*	*5*	*7*

Table 3: Design of experiments

Trial No.	pH	Temp. (°C)	Nitrogen source (1%)	Waterhyacinth (%w/v)	Tween 80 (v/v %)	Inoculum (%)	Time of incubation	Rotation (rpm)
1	3	30	YE	7.5	0.5	5	3	0
2	5	30	Peptone	10	1.5	5	7	0
3	7	30	BE	15	1.5	10	3	0
4	7	30	Peptone	7.5	3	5	5	100
5	3	40	Peptone	15	0.5	7	5	0
6	7	40	BE	15	0.5	5	7	100
7	5	40	BE	7.5	3	7	7	0
8	3	40	YE	7.5	1.5	10	7	100
9	3	30	Peptone	15	3	10	7	150
10	7	30	YE	10	0.5	7	7	150
11	3	40	BE	10	3	5	3	150
12	7	40	YE	10	3	10	5	0
13	5	40	Peptone	10	0.5	10	3	100
14	5	40	YE	15	1.5	5	5	150
15	5	30	YE	15	3	7	3	100
16	5	30	BE	7.5	0.5	10	5	150
17	3	30	BE	10	1.5	7	5	100
18	7	40	Peptone	7.5	1.5	7	3	150

YE= Yeast extract, BE= Beef extract

Main effects determination by level difference (Average effects of factors and interaction)based on S/N ratio analysis

Column #/Factors	Level 1	Level 2	Level 3	L2-L1
1. Temperature	*30.871*	*30.303*		*-.568*
2.pH	*28.598*	*32.321*	*30.843*	*3.722*
3.Nitrogen source	*31.357*	*29.834*	*30.571*	*-1.523*
4.WH(%)w/v	*30.948*	*29.483*	*31.331*	*-1.465*
5 Tween 80 (%) v/v	*31.144*	*29.539*	*31.079*	*-1.605*
6. Inoculum conc.(%)	*30.264*	*30.395*	*31.103*	*.131*
7.Rotation rpm	*30.237*	*30.133*	*31.391*	*-.104*
8. Incubation time (days)	*30.203*	*31.281*	*30.277*	*1.077*

#	Interacting factor pairs (order based on SI)	Columns	SI(%)	Col	Opt
1	*Inoculum conc.(%X rotation (rpm)*	*6X7*	*96.04*	*1*	*[1, 2]*
2	*Nitrogen source X Tween 80*	*3X5*	*60.13*	*6*	*[1, 2]*
3	*Tween 80 X Incubation time*	*5X8*	*56.75*	*13*	*[3, 2]*
4	*Nitrogen source X Incubation time*	*3X8*	*56.16*	*11*	*[3, 2]*
5	*Temp X Inoculum conc.*	*1X6*	*51.1*	*7*	*[1, 1]*
6	*WH(%) X Tween 80*	*4X5*	*49.23*	*1*	*[3, 1]*
7	*WH(%) X Inoculum conc.*	*4X6*	*48.34*	*2*	*[1, 2]*
8	*Inoculum Conc. X incubation time*	*6X8*	*39.66*	*14*	*[1, 2]*
9	*pH X Incubation time*	*2X8*	*34.07*	*10*	*[2, 2]*

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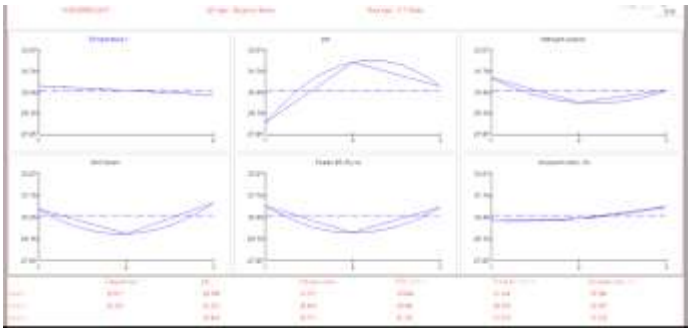
10	Tween 80 X Inoculum conc.	5X6	32.69	3	[1, 1]
11	pH X WH(%)	2X4	32.08	6	[2, 3]

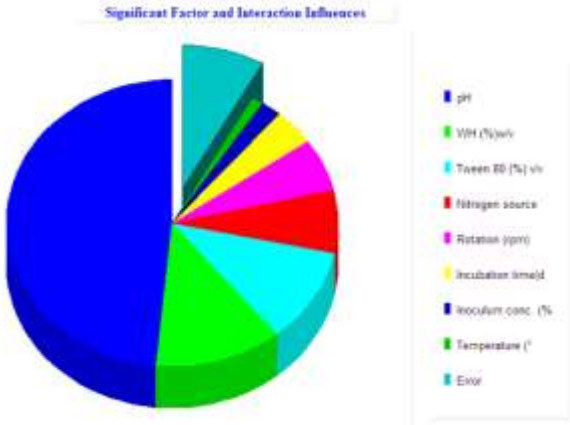
Col #/Factor	DOF(f)	Sum of Sqrs.(S)	Variance(V)	F-Ratio (F)	Pure Sum (S')	Percent P%
1. Temperature	1	1.448	1.448	3.592	1.045	1.224
2.pH	2	42.172	21.086	52.313	41.366	48.468
3.Nitrogen Source	2	6.957	3.478	8.63	6.151	7.207
4.WH(%)w/v	2	11.412	5.706	14.157	10.606	12.427
5.Tween 80 (%) v/v	2	9.908	4.954	12.291	9.102	10.665
6. Inoculum conc.(%)	2	2.442	1.221	3.029	1.636	1.917
7.Rotation rpm	2	5.849	2.924	7.256	5.043	5.909
8. Incubation time (days)	2	4.348	2.174	5.394	3.542	4.15
Other /Error	2	0.805	0.402			8.033
Total	17	85.346				100.00%

Col #/Factor	DOF (f)	Sum of Sqrs.(S)	Variance(V)	F-Ratio (F)	Pure Sum (S')	Percent P%
1. Temperature	-1	-1.448		POOLED	(CL=84.35%)	
2.pH	2	42.172	21.086	52.313	41.366	48.468
3.Nitrogen Source	2	6.957	3.478	8.63	6.151	7.207
4.WH(%) w/v	2	11.412	5.706	14.157	10.606	12.427
5.Tween 80 (%) v/v	2	9.908	4.954	12.291	9.102	10.665
6. Inoculum conc.(%)	-2	-2.442		POOLED	(CL=81.33%)	
7.Rotation rpm	2	5.849	2.924	7.256	5.043	5.909
8. Incubation time (days)	2	4.348	2.174	5.394	3.542	4.15
Other /Error	5	4.695	0.939			11.174
Total	17	85.346				100.00%

Col #/Factor	Level Description	Level	Contribution
1. Temperature	30	1	0.283
2.pH	5	2	1.733
3.Nitrogen Source	YE	1	0.769
4.WH(%)w/v	15	3	0.743
5.Tween 80 (%) v/v	0.5	1	0.556
6. Inoculum conc.(%)	10	3	0.515
7.Rotation rpm	150	3	0.803
8. Incubation time (days)	10	2	0.693
Total contribution from all factors 6.094			
Current grand average of performance... 30.587			
Expected result at optimum condition..... 36.682			



Effect of Rotation on Cellulase production



6.4 Optimum Conditions for Cellulase Production

a) Effect of temperature:

The effect of temperature for optimization of CMCase and FPase two temperature conditions were selected (30^{0}C & 40 ^{0}C). From Figure 10 (a), it can be observed that by increasing the temperature from 30^{0}C to 40 ^{0}C cellulase production increases. Table 6 had shown the main effect of temperature on CMCase production, which was highly effected by temperature whereas FPase production was less affected by temperature. From Table 6 it can be observed that the increase of temperature leads to increase in cellulase production. Result of ANOVA was shown in Table 9. Which includes the sum of squares(s), variance (v), F-ratio (F), Pure sum (s') and percent (P). For temperature at Degree of factor 1, sum of squares 9.2, variance 9.2, F-ratio12.2, the percent contribution obtained was 21 % for CMCase whereas for

FPase at Degree of factor 1, sum of squares 0.9, variance 0.9, F-ratio 3.4, the percent contribution obtained was 5.3 %. Thus it can be concluded that temperature is one of the most influencing factor for CMCase production. While FPase production is effected by the temperature. Similar work was done by the **Despande *et al.,*** *2009; he reported the maximum cellulose activity at temperature 30ºC. Temperature has a great influence on enzyme production. If temperature is too high, microorganisms grow faster but enzyme production is low and if temperature is low, microbial growth is slow, resulting in long production cycle, so optimization of temperature is a necessity.* **Guowei *et al.,* 2011** *in their study used different incubation temperature (26°C, 28°C, 30°C, 35°C and 40°C.) for enzyme production. The activity of CMCase and FPase first increased up to certain value and then decreased. The activity of CMCase increased from 236.09U/g at 26°C to377.20U/g at 30°C and then decreased to 21.61U/g at 40°C. The activity of FPase increased from 37.33U/g at 26°C to 92.16U/g at 30°C and then decreased to 5.48U/g at 40°C.The optimal incubation temperature was 30°C for CMCase and FPase.* **Nochaure *et al.,* 1993** *studied the effect of temperature on cellulase enzyme production by the A. Niger and Trichoderma reesei in the range of 20 to 85 ± 2°C.The optimal temperature for exoglucanase (1.95 U/mL) and endoglucanase activity (1.88 U/mL) A. niger was 40°C and 50°C, while the optimum temperature for β-glucosidase activity was between 45°C and 55°C.*

b) Effect of pH

The pH selected 3, 5, &7 pH is one of the most important contributor factors after the temperature in cellulase production. On increasing the pH from 3-5, there was a sharp increase in the enzyme production, after further increase in pH from 5-7 enzyme production ceases rapidly. This can be conformed from Fig.10 (b) pH interacts with many factors such as (SI%38) especially with rotation for CMCase production while for FPase,pH interacts with WHwith (SI%56). High dissociation of ions during rotation helps in more mass transport. According to Table 7 FPase productions were highly dependents on nitrogen source, temp and substrate concentration. For CMCase the pair of factors that affects production are Tween80 (SI%12), incubation time (SI%25).As per ANOVA analysis, pH is second highest contributor factor in CMCase production and first highest contributor factor in FPase production with F-ratio 6 & 5.2 respectively. This data can be conformed from Fig 11. pH plays an important role in enzyme production since the activity of the enzyme depends on optimum pH, so optimization of pH is one of the important factor for enzyme production. The influence of pH on enzyme production was studied by **Chung *et al.,* 2010.***They took different pH in the range from 3.0-9.0. Their results showed maximum production of exoglucanase was 1.76 & 2.18 U/mL, while endoglucanase was 1.25 & 1.95 U/mL, and β-glucosidase was 1.44 & 1.71 U/mL by Aspergillus niger and Trichoderma reesei at pH 6-7 and In another study by* **Liu *et al.,* 2007** *where they used waste from vinegar industry as a substrate for production of cellulase by Trichoderma choningii AS3.4262. The Fpase activity obtained was 6.90 IU/g of SDM (substrate dry matter) and CMCase activity 23.76 IU/g. Similar work had been performed by* **Liu *et al.,* 2007** *cellulase were produced by using T.choningii AS3.4262. The activity of FPase and CMCase was reported as 6.90 IU/g and 23.76 IU/g after 48 hrs with pH 5.0.* **Karmakar *et al.,* in 2011** *reported cellulase production using WHusing Rhizopus oryzae MTCC 9642 in submerged and solid state fermentation. Various parameters has been studied such as substrate conc., temp and pH were optimized. The best FPase activity from submerged fermentation was at substrate conc. 1.25%, pH 7.32 and temp 25 ºC while as in SSF mode the best enzyme production was obtained at substrate conc. 0.5%, pH 6.0 and temp 18 ºC.*

c) Effect of Tween 80

Effect of Tween 80 was studied at 3 levels 0.5%, 1.5% and 3% as shown in Fig 10 (c).After addition of 1.5% of Tween 80, CMCase production ceases while FPase production does not .This may be due to effect of other factors interacting with Tween 80 such as rotation speed (SI % 54), inoculums percent (SI% 49), nitrogen source (SI%46). (Table 7). Similar work had done by the **Sharhriarinour *et al.,* 2011***. He studied the effect of Tween 80 on cellulase production, according to results cellulase production was enhanced with addition of Tween 80 in culture (Tween 80 at a conc. of 2 ml/l). Tween 80 is the surfactant which increases mass transport by reduction in viscosity. As a result, at lower concentration, this may be an important additive, whereas at higher concentration, this may not be supportive for high enzyme production because of toxicity and reduced mass transport due to bubble formation.*

d) Effect of water hyacinth

Effect of Water hyacinth was studied at concentration 3 levels was 5%, 7-5% & 10% shown in Fig 10 (d). On increasing the conc. of WHfrom Level1 to Level 2 (7.5-10% w/v), there is a rapid increases in CMCase and FPase production, after that the enzyme production decreases. Water hyacinth interacts with other factors, which is depicted in Table 7. The other interacting factors are incubation time (SI%44) and (SI%25) for CMCase and FPase respectively. Nitrogen source (SI %23), (SI%31) for FPase, with Tween 80 (SI%60 & SI %42), WHand inoculum (SI%82 & 29). This indicates that for hydrolysis of water hyacinth, the most active enzyme is CMCase which requires high percentage of inoculum. Other factors which effects hydrolysis of WHis incubation time (at least 5 days) to start the process. Since enzyme is protein which requires high %age of nitrogen supply, thus effect of nitrogen is around (SI%23) in CMCase while as in FPase it is (SI%31). Water hyacinth (Eichhornia crassipes), an aquatic weed creates a lot of ecological and socio-economic problems to water bodies **(Takasawa *et al.,* 1986)***.Various workers has used WHfor production of fuel and chemicals* **(Burton *et al.,* 2005).** *Thus, WH(Eichhornia crassipes) may prove a highly economical substrate for cellulase production* **(Osei-agyemang *et al.,* 2002).** *According to various reports, WHhas been found rich in hemicellulose followed by cellulose and other components impregnated with the lignin. Water hyacinth may prove highly beneficial for enzyme production since they are rich in protein and various nitrogen sources besides carbohydrates* **(Ghosh, 1981)***. Thus beside cellulases, they are also good substrate for ethanol and organic acids production* **(Zha *et al.,* 2008).** *Another advantages is WHas economical substrate & that they are*

readily available round the year.According to **mukhopadhyay *et al.,* 1999** *increase in WHaround 2.6%.There was 4% increase in cellulase production and ratio of β-glucosidase to FPase was higher 6%. Ammonium sulphate for pepton was best nitrogen source for cellulase production* **Despande *et al.,* 2009** *conducted work for cellulase production by using Water hyacinth as a substrate. Parameter studied for substrate pre-treatment was substrate concentration, initial medium pH, mode of incubation, temperature. Maximum cellulase activity reported was 0.22IU/mL after 15 days. Mass transport of WH(from external medium to cell), the most important factor observed was rotation, incubation time, Tween 80 which collectively is responsible for giving the high result of CMCase production, while almost similar condition prevails for FPase production. Further various fungal strain Aspergillus flavus, Aspergillus niger, Trichoderma reesei MTCC164 have been reported for cellulase production* **(Ismail *et al.,* 1995)**

e) Effect of nitrogen source

Different nitrogen sources were used for cellulase production such as yeast extract, beef extract, peptone at 1% (w/v).Selection of organic nitrogen was based on the fact that at very low concentration nitrogen has different effect over enzyme production. From Fig 10(e) all three nitrogen have almost equal effect thus no detectable change has been observed by changing from Level 1 to Level 3.CMCase production is positively affected by organic addition of nitrogen (0.247) while FPase production is effected by besides main effects there are various other factors that affect nitrogen utilization and mass transport as observed in Table 7. **Ali *et al.,* 2008**, *used WHblend for production of cellulases using mixture of Microbes such as A. niger and A. nidulans in Czapek-Dox medium. According to their report, the maximum enzyme activity was found at following conditions such as temp 35 ºC, pH 7.0, sodium nitrate was found to be as best nitrogen source and 7 & 3 days under static and shaker conditions respectively for A. niger and at 30 ºC, pH 7.0, sodium nitrate as nitrogen source and 7 & 4 days under static and shaker conditions respectively for A. nidulans. Similar work had done by the* **Leynd *et al.,* 2002** *repored maximum production of cellulase enzyme by using 1.0% peptone, beef extract and exoglucanse produced was 1.79 μ/ml while endoglucanse was 1.48 μ/ml and β-glucosidase was 1.92 μ/ml by T. ressei.For CMCase production, incubation time, rotation at (SI%51 and 49), Tween 80 46% for CMCase production. Nitrogen accumulation is effected by similar condition for FPase production at different SI conditions. ANOVA analysis confirmed the fact that organic nitrogen has 4% of overall contribution of different factors at F-ratio 2.1, while for FPase very low effect of organic nitrogen has been observed .this can be conformed form Fig 11.*

f) Effect of incubation time.

The effect of incubation time was selected for study was 3, 5 & 7 days in hydrolysis of WHincubation time plays an important role. On increasing the time from level1 and level 2 in both the enzyme CMCase and FPase. Individually there is less effect of incubation time in CMCase production (as depicted by Fig 10(f), from Table 7). It has been observed that incubation time is also involve not only in hydrolysis of WH(44% SI) but also accumulation of organic nitrogen at (SI %51) other factors such as pH, Tween 80 has little contribution towards enzyme production (SI%25).Almost similar condition prevails for CMCase and FPase production.

Devi and Kumar, 2012, *optimized the cellulase production from Bacillus cereus MRK1 and performed its Bio stoning activity. Different factors such as incubation period, temperature, pH and effect of carbon and nitrogen sources were optimized for maximum yield of the enzyme. Initial optimization process showed pH 8, 32 °C, xylan and yeast extract favouring enzyme production. The test strain showed its ability to secrete cellulase around 102 IU/ml when it was grown in paper sludge supplemented medium. Cellulase production was studied by* **Omojasola *et al.,* 2008** *using pineapple waste as substrate using A. niger, Trichoderma longibrachiatum, and Saccharomyces cerevisiae as inoculum. The various factors optimized were time, pH, substrate conc. inoculum size and temp. Out of the above mentioned cultures, Trichoderma longibrachiatum produced higher amount of glucose (0.92 mg/0.5 ml) at pH 4.5 and temperature of 45ºC on day 7th day of fermentation.*

Deaming *et al.,* 2008 *studied the cellulase enzyme activity from T. reesei up to 6 days. The maximum yield of exoglucanase, and endoglucoanase activity was obtained after 5 days. After ANOVA analysis Table 8 incubation time has 5.8% contribution of factors F-ratio 2.57 for CMCase production while for FPase; contribution is almost 15.9% at F-ratio 4.7 this can be conformed from Table 9.*

g) Effect of Inoculum (%)

The effect concentration of inoculums selected was 5, 7 & 10 for CMCase and FPase production, with the increase in concentration of inoculums had little effect on production shown in Fig 10 (g). According to ANOVA Table 8, it contributes only 0.7 % for CMCase and around 5% for FPase and which can be conformed from Fig 11. **Shikai *et al.,* 2013** *conducted a similar work, result shown that when the concentration of inoculums was increased then the activity of CMCase assay increased from 355.06 U/g at 0.5% to 386.47 U/g at 2.5%.*

h) Effect of rotation

The individual effect of rotation condition selected for study was 0, 100 and 150 rpm on enzyme production. In Fig 10 (h) on increasing the rotation speed, continuous increase in CMCase production from level 1 to level 3 while for FPase after level 2 is a sharp decrease in FPase production. Rotation has positive effect over the carbon substrate utilization, dissolution of various media components and helps in efficient aeration and nutrient transport. It also helps in maintenance of uniform pH and temperature. Besides this, they are also responsible for product removal at uniform rate from cell wall. The similar effect can be observed in both the enzymes production. For CMCase production rotation has 3rd highest SI impact 54 % and for FPase 2nd highest SI impact was observed 61%.

Sarkar *et al.,* 2012 *reported the effect of rotation on cellulase production. At rotational speed 120 rpm and temperature 37ºC CMCase and FPase activity was increased in cellulase production. From ANOVA analysis (Table 8) rotation has 5 % contribution for CMCase production while*

it is 10% for FPase production. The same contributed by Fig 11.

FPase assay	Av.OD (550 nm)	CMCase assay	Av. OD(550 nm)
Trial 1	0.256	Trial 1	0.284
Trial 2	0.262	Trial 2	0.363
Trial 3	0.485	Trial 3	0.454
Trial 4	0.557	Trial 4	0.597
Trial 5	0.639	Trial 5	0.683
Trial 6	0.586	Trial 6	0.543
Trial 7	0.957	Trial 7	0.896
Trial 8	0.262	Trial 8	0.766
Trial 9	0.331	Trial 9	0.526
Trial 10	0.392	Trial 10	0.492
Trial 11	0.285	Trial 11	0.405
Trial 12	0.355	Trial 12	0.334
Trial 13	0.238	Trial 13	0.389
Trial 14	0.296	Trial 14	0.326
Trial 15	0.312	Trial 15	0.349
Trial 16	0.454	Trial 16	0.264
Trial 17	0.426	Trial 17	0.218
Trial 18	0.434	Trial 18	0.246

Tabl 6: Main effects determination by level difference (Average effects of factors and interaction)

Column/factors	L2-L1	L2-L1
Temperature (°C)	1.435	0.217
pH	1.408	0.353
Tween 80 (%)	0.609	-0.321
Water hyacinth (%) w/v	0.714	0.319
Nitrogen source	0.247	-0.411
Incubation time (days)	-0.248	-0.988
Inoculum (%)	-0.515	-0.723
Rotation (rpm)	-0.246	-0.026

Conclusion and Future Scope

In our work increasing 1.5% WHresulted in increase in FPase 76% as comparably to **Despande *et al.*, 2009.***while CMCase activity as 50% higher while FPase was 87 % higher. This is because of increase mass transport when media were supplied with Tween 80-1.5%, peptone 1%, rotation-100 rpm; inoculums level -7.5%, incubation time-7days and WH-7.5% in SSF mode. Various chemical pre-treatment were done such as HCl, NaOH, and Lactic acid along with steam explosion. Out of all the experiments performed, NaOH (8%) gave the best result for pre-treatment. While as other results were not significant. Trichoderma reesei selected with WHfor cellulase production. Optimization the effective conditions for production of CMCase temp-40 °C, pH-5, tween80 3%, WH7.5%, nitrogen source 1% as peptone, incubation time 7days, inoculum 5% at rotation 100 rpm for FPase production only two condition was difference inoculum was higher (10%) and rotation speed was 150 rpm. Production of cellulase for CMCase was 52% more increase activity observed after media optimization and similar for FPase was 84% increase activity observed after media optimization.*

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