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Bagasse a lignocellulosic substrate is resistant to microbial attack and is considered to be poor in digestibility. The presence of lignin in bagasse makes it unfit as animal feed and several workers have given the methods of removal of lignin by chemical, physical and microbial methods for rendering it suitable for single cell protein (S.C.P.) production. Enzymes ligninase-laccase and cellulolytic activities of organisms were found to be responsible in such decompositions. Bacteria are incapable of attacking the lignocelluloses and moulds belonging to *Trichoderma*, *Penicillium*, *Polyporous* spp. have been reported in such decompositions.

The present dissertation deals with the isolation, purification, morphology and growth characteristics of two basidiomycetes class mould isolates capable of converting lignocellulosic substrate-bagasse to protein and production of cellulases in the culture filtrate.

Basidiomycetes Strains:

The cultures were isolated from biomanure and
bioliquid samples of Institute biogass plant using lignocellulosic substrate (Corn cob, rice husk, bagasse etc) as raw material for methane generation. The mould contaminants obtained from biomanure samples were incubated at 30°C in petridishes containing bambo paper pulp admixed with bagasse powder (8:2) in (minimal) Mandel's-weber basal salt medium for about a month in humid atmosphere.

The mould cultures were purified by conventional planting techniques and gave three colonies designated as BC_{1}, BH_{1} and BW_{1} having ligninolytic and cellulolytic activities. The cultures showed Bavendam's test of phenol oxidases in the decomposition of lignin of bagasse. In further morphological and physiological studies and growth on bagasse, only two cultures were found good e.g. BH_{1} and BW_{1} out of three isolates showing 66.6% and 55.5% total carbohydrate (T.C.) decomposition respectively and having high content of protein in biomass formed (Tab.1b). The cultures were characterized according to taxonomic data based on Alexopoulos and found to belong to Basidiomycetes class and Polyporous genus (Tab.5). These two cultures were taken in these fermentation and enzyme studies.
Norkran's medium was found best for growth and enzyme production among the three media tried namely Kirk and Kelmen's, Mandel's-Weber media. BH<sub>1</sub> was capable of giving high salicinase and ligninase activities and BW<sub>1</sub> high CMCase and FPD activities. Cellulases reached maximum in 3-5 days at pH optima of 4.8 to 5.0 and ligninase in 7 to 12 days of incubation (Table 3).

Washed bagass was found superior as substrate for FPD and salicinase in BH<sub>1</sub> culture and salicinase in BW<sub>1</sub> culture. Alkali-treated bagasse was best for CMCase activity in both cultures, whereas whatman filter paper No.1 gave low activities in both cultures. Salicinase activity was higher in BH<sub>1</sub> as compared to BW<sub>1</sub> and cellulose powder acted as good substrate, especially showing high CMCase in BW<sub>1</sub> culture (Table 4).

**Pretreatment of bagasse:**

Bagasse powder (65mesh) treated with different physical and chemical agents indicated that lignin removal was maximum in peracetic acid treatment and minimum in sulphite treatment method.

Cellulolytic activities reached maximum in 3-5
days of incubation in all pretreated substrates along with the control (Fig. 16, 17). CMCase and FPD were more in BW\textsubscript{1} whereas salicinase was more in BH\textsubscript{1}. Oxidizing agents and lime treatments tried, gave good results in protein production (except chlorite in BW\textsubscript{1}) but not in cellulase production. Dioxane treatment gave maximum decomposition of total carbohydrate but showed low protein production in both BH\textsubscript{1} and BW\textsubscript{1} cultures. Steam and sulphite treatment gave similar results in both cultures showing improvement in CMCase and FPD activities as compared to other treatment.

**Optimization of parameters for cellulases in submerged fermentation (S.F.):**

Using whole washed bagasse powder (65mesh) at 1\% concentration in Norkran's medium at pH 5.0 to 5.5 and inoculum size of 6 discs (6\text{mm dia}) of mycelium from malt agar grown plates (72 hrs.) was found best in the growth and enzyme production. Washed bagasse could not be replaced by unwashed bagasse since enzyme production was less. Sucrose addition of 0.1\% to unwashed bagasse showed some increase in the production of cellulases by both cultures but lactose 0.1\% increased only CMCase and FPD of BW\textsubscript{1} culture and it inhibited cellulases of BH\textsubscript{1} culture.
Using wheat bran (20%) in admixture with bagasse, increased all the cellulases in BH₁ by 30-50% but in BW₁ it inhibited the release of enzymes.

Addition of sugars i.e., sucrose, xylose or lactose and their combination at 0.1% and 0.2% concentration improved CMCase and salicinase without affecting FPD in BH₁ culture.

In BW₁, effect of these above carbohydrates improved the release of enzymes. Sucrose and lactose at 0.1% concentration did not show much effect and xylose at 0.1% tripled the salicinase. Combined effect of sucrose and lactose at concentrations 0.1% and 0.2% each increased all cellulolytic activities in both the cultures (Fig.20b).

Among vitamins (Biotin, Niacin etc.) at 10 mg/l did not significantly affect the enzyme release in both BH₁ and BW₁ cultures (Tab.11, Fig.21).

Among the amino acids added at 100 mg/l concentration, Aspargine and Tyrosine showed inhibition of salicinase and valine stimulated CMCase and salicinase of BH₁ culture. Serine, Tyrosine, valine stimulated
salicinase whereas Lysine stimulated CMCase and salicinase of BW\textsubscript{1} culture.

Tween 80 (1\%) was found activator for salicinase in BH\textsubscript{1} and BW\textsubscript{1} cultures. Copper at (100mg/l) stimulated salicinase of BW\textsubscript{1} culture.

Adipic acid and Butylester at 0.5\% concentration were found inhibitory for cellulases of both BH\textsubscript{1} and BW\textsubscript{1} cultures but Butylester at 0.1\% and below acted as good stimulator for production of cellulases in both BH\textsubscript{1} and BW\textsubscript{1} cultures. Lauric acid Palmitic but not stearic at 0.05\% concentration acted as stimulants of CMCase in BH\textsubscript{1} culture and in BW\textsubscript{1} Stearic acid, Lauric and Palmitic acid at 0.5\% acted as good stimulator of cellulases especially CMCase and FPD. Lowering of Lauric acid concentration 0.5\% was effective for increase of cellulases in both BH\textsubscript{1} and BW\textsubscript{1} cultures (Fig.24a,b).

All the phenolic compounds i.e., Tannic acid, Gallic acid and Resorcinol at 100mg/l concentration acted as stimulator of cellulases in both the cultures BH\textsubscript{1} and BW\textsubscript{1}.

Charcoal addition (1g/l) helped in controlling cellulase activity of culture filtrate beyond 7-9 days in
both BH$_1$ and BW$_1$ which otherwise used to drop very fast.

Different alcohols at 1% concentration inhibited FPD activity of BH$_1$ culture and at later stages on 5th day all cellulase activities were reduced.

Peptone 0.1% and glucose 1% stimulated all cellulolytic activities in both BH$_1$ and BW$_1$ cultures.

**Solid State Fermentation (S.S.F.):**

15-20 days fermentation period using 6 discs of 6 mm dia inoculum was found more suitable for cellulase production in (S.S.F.).

Washed bagasse was found best for cellulase production as compared to unwashed bagasse as substrate in both BH$_1$ and BW$_1$ cultures. Tween 80 (1%) concentration doubled the CMCase activity without affecting FPD and salicinase in BH$_1$ culture, whereas in BW$_1$ it increased CMCase and salicinase by about 3 times of control with slight stimulation of FPD activity (Tab.21, Fig.27).

Cu$^{++}$ concentration from 0.01% to 0.04% showed
stimulation of only CMCase in BH₁ culture and salicinase in BW₁ culture was almost doubled.

Lactose, Sucrose and Xylose, sucrose and lactose mixtures at 0.2% and 0.4% each showed improvement in all cellulases in BH₁ culture especially CMCase and salicinase which were more than doubled. Xylan 20% as substrate was also found good for release of cellulases in S.S.F. (Tab.22a, b, Fig.28a, b).

Effect of above added sugars in BW₁ showed slight stimulation showing about 20-25% increase in cellulase activities. Lactose+ Sucrose at 0.2% and 0.4% each showed appreciable stimulation of salicinase. Xylan at 20% was found good for release of cellulases especially FPD activity.

Among vitamins added at (10mg/l) except Biotin showed increase of CMCase in both BH₁ and BW₁ cultures.

Addition of $\beta$-glycerophosphate, metaphosphate and pyrophosphate showed stimulation of CMCase and salicinase of BH₁ and BW₁ cultures.

Addition of Purine, nucleoside and nucleotide
doubled the CMCase in BH\textsubscript{1} culture whereas in BW\textsubscript{1} increase in CMCase was only 20%.

Phenolic compounds such as Tannic acid, gallic acid and Resorcinol increased cellulases in BH\textsubscript{1} and BW\textsubscript{1} cultures by about 50-100% and effect of these compounds on salicinase production in both the cultures was maximum.

**Mixed Culture Fermentation (S.F.):**

Studies were carried out for better and maximum utilization of bagasse employing mixed culture fermentation technique. Isolated mould culture BH\textsubscript{1} was mixed with BW\textsubscript{1} in different combinations to study their effect on cellulase production. Cellulase activities were maximum on 5th day.

Cellulolytic activities in culture filtrate were best when BH\textsubscript{1} was grown first and BW\textsubscript{1} was inoculated on 3rd day. Next to this was simultaneous growth of BH\textsubscript{1} and BW\textsubscript{1}. Cellulase activities were least when BW\textsubscript{1} was grown first and BH\textsubscript{1} was inoculated on 3rd day (Tab.27).

Ligninase was high when BH\textsubscript{1} was grown first and BW\textsubscript{1} was added on 3rd day and was least when BH\textsubscript{1} and BW\textsubscript{1}
were grown simultaneously (Tab.27). Maximum ligninase production was on 12th day (Fig.33).

Protein formed was maximum 22% when BH₁ and BW₁ were grown simultaneously, 20% protein was formed when BH₁ was grown first and BW₁ was added on 3rd day. Least protein was formed (7%) when BW₁ was grown first and BH₁ was added on 3rd day (Tab.27).

In mixed culture cultivation using simultaneous growth, gave maximum biomass formation with maximum total carbohydrate decomposition. Total carbohydrate decomposition was maximum when BW₁ was grown first and then BH₁ was added but protein/biomass formed was low.

Studies on cellulases and Ligninase parameters, Enzyme immobilization on bagasse substrate during S.F., mixed culture and S.S.F. of BH₁ and BW₁ cultures:

Cellulase purification:

The cellulases released in culture filtrate were purified by salt, solvent and their combination. Fractionation with 80% was found best. Similarly acetone 80% fraction also produced good results and could be
stored for about a month with 50% loss in activities. The system of fractionation using combination of ammonium sulfate and acetone was not found good (Tab.31).

Effect of Sugars on cellulases:

Sucrose and Lactose at 0.1% concentration acted as activators of all cellulolytic enzymes of both BH₁ and BW₁ cultures.

Thermal Stability and heating effect:

Preheat treatment of above enzymes at 50°C and 70°C for 10 min. and 40 min. showed inhibition of CMCase, FPD and Salicinase respectively in both BH₁ and BW₁ cultures but compared to FPD and salicinase in BH₁ culture CMCase loss was less. In BW₁ all the cellulases were affected. Dialysis of enzymes showed improvement in cellulase activities. Heat treatment of dialyzed enzyme as above showed activation in CMCase with less loss in FPD and salicinase, both BH₁ and BW₁ culture (Tab.32).

Ligninase activity in culture filtrate of BH₁, BW₁ and mixed culture BH₁ + BW₁:

Culture filtrates of BH₁, BW₁ and BH₁ + BW₁ showed
appreciable ligninase activity. The ligninase activity was proportional to concentration of enzyme in filtrate and 50 μM guaiacol substrate was found optimum for activity with pH optima of 5.6 (Fig. 39, 40). The ligninase activity showed almost linear relationship up to 6hrs. of reaction at 30°C (except in BW₁ culture). BH₁ showed maximum ligninase activity as compared to BW₁ and BH₁+ BW₁ cultures.

All the oxidizing agents tried showed 25 to 100% inhibition except in the action of potassium ferricyanide in BH₁ culture whereas in BW₁ all above oxidizing agents showed 100% inhibition (Tab. 33).

Adsorbed/Immobilized Enzyme (Cellulase):

Cultures BH₁ and BW₁ grown on bagasse under submerged fermentation, mixed culture and solid state fermentation for the production of cellulase enzyme in residual substrate left after complete extraction of enzymes was high, indicating adsorption of cellulases on bagasse. CMCase and salicinase activities adsorbed on bagasse of fermentation (S.F.) in both the BH₁ and BW₁ cultures. FPD activity adsorbed in bagasse was maximum on 3rd day in BH₁ and in BW₁ it was on 5th day (Fig. 34).
Salicinase activity in adsorbed bagasse of mixed culture, submerged fermentation were more than double of normal cultures (control) fermentation, especially in the combination system when BH₁ was grown first and then BW₁ was added. Simultaneous growth of BH₁ and BW₁ cultures showed good results as compared to controls for adsorption of enzymes (Fig. 35).

In S.S.F. adsorbed cellulase activities gradually increased in bagasse substrate from I to V week in both BH₁ and BW₁ cultures (Fig. 36).

Reuse of adsorbed bagasse as cellulase Enzyme:

Reuse of adsorbed bagasse as enzyme source could give only 3 cycles (S.F.) showing fast drop (Fig. 37). Whereas in S.S.F. adsorbed cellulase gave 6 cycles with gradual decrease.

The adsorbed system of S.S.F could be preserved with lactose 0.5% in both cultures and glycerol 50% was also found good for both cultures. Different treatments tried to preserve adsorbed cellulase of S.F., Toluene, acetone and PVA 3% could be used as preservatives, whereas acetone was found best preservative and also showed
stimulation in CMCase and salicinase in both the cultures (Tab.29).

Desorption of adsorbed cellulases (S.F.):

In BH culture all the buffers (acetate, citrate and phosphate) tried favoured the desorption of adsorbed cellulases from residual bagasse. They also showed activation in CMCase and FPD but not in salicinase. Maximum release of CMCase and FPD was found by using 0.5M NaCl. Addition of azide, triton, urea in buffer helped in the release of CMCase but not FPD, possibly due to their inhibition on FPD activity. Ultrasonication released only salicinase and was high in 5 min. of sonication, increasing the time decreased the release (Tab.30a).

In BW cultures none of the buffers tried favoured desorption. Increasing the molarity and salt concentration (NaCl) slightly helped in the release of enzymes. Citrate and phosphate buffer showed some release of enzymes. Addition of azide, urea and triton 100, in buffer acted as enzyme inhibitors. Ultrasonication released only salicinase in about 20 minutes of sonication (Tab.30 b).
Saccharification of different cellulosic substrate using adsorbed enzymes of submerged fermentation:

In saccharification, enzyme—substrate (10:1) were taken in 40 ml acetate buffer pH 4.8 and incubated at 50°C and 70°C under stirring condition and the sugar released was estimated. Above studies showed that BW₁ culture enzyme showed best results as compared to BH₁ giving about 22% to 27% hydrolysis of different cellulosic substrates in 24 hrs. reaction in single step or with one cycle (Tab.34).

Chromatographic examination of enzyme reaction product:

BH₁ and BW₁ purified cellulase reaction mixtures with high cellulolytic activities showed the presence of glucose as main sugar product in the reaction mixtures. Traces of cellobiose was also observed in the hydrolysates of bagasse and salicin (Fig.11).

The fungal mycelial protein digest showed the presence of almost all the essential amino acids in the hydrolysates of BH₁ and BW₁ cultures (Fig.13).