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Cellulolytic Enzyme Activity of Two *Pleurotus* Species Grown on Different Agricultural Wastes

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SOME different agricultural wastes have been tested for their ability to stimulate the cellulolytic activity by *Pleurotus pulmonarius* and *Pleurotus columbinus*. Pea pods and cotton stems were the best wastes used for production of cellulolytic enzymes (Avicellase, Carboxymethyl cellulase, and β-glucasidase) by both fungi when added separately to the liquid medium as carbon sources. Bean pods and sugarcane bagasse supported high production of cellulolytic enzymes by *P. pulmonarius* and *P. columbinus* respectively. Addition of pea pods and cotton stem separately to the liquid medium containing wheat or rice straw at concentration 4% and 3% respectively , enhanced the production of all enzymes produced by the two fungi. Avicellase was the enzyme produced in highest concentration. It has been chosen to optimize enzyme production. The highest Avicellase activity was obtained after seven and eight days incubation for *P.pulmonarius* and *P.columbinus* respectively. The pH optima for Avicellase activity were 6 and 5.5 for *P. pulmonarius* and *P. columbinus* respectively. Among the nitrogen sources examined, potassium nitrate and asparagine gave the best Avicellase production by *P.pulmonarius* and *P.columbinus* respectively, followed by ammonium chloride and ammonium nitrate for both. Increasing nitrogen level up to 3.2gN/l enhanced Avicellase production. A sharp decrease in Avicellase production after this concentration has been recorded.

Enormous amounts of lignocellulosic materials have been constantly produced in nature by photosynthesis with an estimated annual production of about 23 million tonnes of agricultural residues in Egypt (Hamdan, 1990) which makes them an important potential resource for industry and agriculture.

Waste cellulosic materials are categorized by Halpern (1981) as municipal solid wastes, industrial wastes as paper mill effluents, sawdust and sludge, and agricultural wastes as grasses, straws, bagasse, hull, stalks and pomaces. In Egypt, most of these materials are currently burned, buried, or otherwise discarded without recycling, or used quite inefficiently.

Some fungi were reported to be very effective in biodegradation of cellulosic wastes (Nour El-Dein, 1992).

Mushroom production is a currently available economic process for utilisation of lignocellulosic residues (Wood, 1985). Mushrooms are known to produce a variety of extracellular enzymes responsible for degradation of cellulose, hemicellulose and lignin fractions of lignocellulosic materials (Wood *et al.*, 1988 & 1990 and El-Fallal, 2001). Enzymatic degradation of straw provides nutrients for mushroom mycelial growth.

Oyster mushroom cultivation has been introduced in Egypt in the last two decades (Mohamed & El-Kattan, 1989). *Pleurotus* spp have been proved to degrade lignocellulosic wastes (Lang *et al.*, 1996; Tan & Wahab, 1997 and Lang *et al.*, 1998).

This paper describes the effect of different agricultural wastes on production of cellulases by *Pleurotus columbinus* and *Pleurotus pulmonarius* and determine the highest yields of cellulases under different controlled conditions.

Experimental

Material and methods

Pleurotus columbinus Quel.ap.Bres and *Pleurotus pulmonarius* Fr. were obtained from consulative Comet Company of mushroom cultivation-Egypt (CCCM). These basidiomycetes were maintained
at 30°C on 1.5% MA slopes.

Media and growth condition

Pleurotus spp were grown in 250 ml Erlenmeyer flasks containing 50 ml of Czapek-Dox medium. This medium consisted of (%w/v): sucrose, 3; sodium nitrate, 2, potassium dihydrogen phosphate, 1, magnesium sulphate, 0.05, potassium chloride, 0.5, ferrous sulphate, 0.01. The asparagine-nitrogen was used at the best concentration 3.2 gN/L (El-Fallal *et al* in press). pH was adjusted to 7. The carbon source was added as 1% of different agricultural wastes: pea pods, bean pods, sugarcane bagasse, cotton stems, wheat bran, pericarp of palm, corn stem, rice husk or sawdust. The main mushroom bed materials were wheat straw and paddy straw. These wastes were oven dried at 80°C for 24 hr and milled using Grinding Mill Model A_{10} IKA and passed through 0.3mm sieves. The flasks were inoculated with two 6-mm agar discs of the two fungi cut from five-day-old culture and incubated statically at 30°C for 16 days.

After the incubation period the wastes and mycelia were filtered off and the filtrate stored at 0°C prior to enzymes assays.

Assay of cellulases

Activities of C_1(Avicellase), C_x (Carboxymethyl cellulase) and C_2 (β-glucasidase) were determined colourimetrically by the method of Mandles *et al.* (1976). One ml of culture filtrate was added to 1 ml of 0.05M citrate buffer, pH 4.8, containing 10mg of crystalline cellulose (for C_1 estimation), carboxymethyl cellulose (for C_x estimation). After incubation for 30 miutes at 40° C, the reactions were terminated and reducing sugar released was determined by the dinitrosalicylic acid method (Miller, 1959). The tubes were placed in a boiling water bath for 15 min then cooled to room temperature and the absorbance was read at 575nm.

One unit of cellulase activity (C_1, C_x or C_2) was defined as the a bility o f producing the reducing sugars equivalent to 1 μ mole of glucose per minute.The absorbance was measured for the activities of all enzymes on Model 340–spectrophotometer Sequoia – Turner– Corporation California.

Optimization of avicellase

The effect of incubation period, changing of initial pH, different nitrogen sources and nitrogen concentrations have been tested using the wastes that stimulated the highest Avicellase activity, the highest cellulase activity, produced by both fungi.

The results of the experiments were statistically analyzed using Least Significant Difference (L.S.D) test at 5% level of probability (Snedecor & Cochran, 1969).

Results

Preliminary experiments were carried out to investigate the optimum growth conditions of both *Pleurotus* spp. The best results achieved have been used in the next experiments.

Results represented in Tables 1 & 2 showed that, of the eleven tested wastes pea pods, sugarcane bagasse, cotton stems, wheat bran and bean pods were the best wastes for stimulating production of cellulolytic enzymes by both *Pleurotus* spp.

It is obvious from Table 1 that enzymatic activities in the filtrate were highest for *P. pulmonarius* in pea pods-amended flasks followed by sugarcane bagasse and cotton stem. Although there were no significant difference between them for stimulation of C_1 and Cx, cotton stem was significantly more stimulatory to C_2 activity than was sugarcane bagasse. Also bean pods stimulated both C_1 and C_x with no significant difference from cotton stems. Wheat bran moderately supported production of C_2 followed by dates seeds.

TABLE 1. Effect of different wastes on production of cellulolytic enzymes by *P. pulmonarius*.

Wastes	Enzyme activities (units / ml)		
	C_1	C_x	C_2
Pea pods	0.83	0.48	0.78
Sugarcane bagasse	0.26 b	0.21 b	0.54
Cotton stem	0.28 ba	0.17 ab	0.70
Wheat bran	0.25 ba	0.16 a	0.34
Rice straw	0.07 c	0.02 c	0.2a
Date – seeds	0.05 c	0.09 d	0.28
Corn stem	0.05 c	0.08 d	0.26 a
Wheat straw	0.03 c	0.05 c	0.20 b
Rice husk	0.03 c	0.02 c	0.18
Sawdust	0	0	0
Bean pods	0.21 a	0.20 ab	0.22 b

* The values represent the mean of 3 replicates.
* a,b,c,d, means accompanied by the same letters are not significantly different at (p= 0.05) .

Table 2 shows that pea pods and bean pods and cotton stem efficiently stimulated C_1 activity by *P. columbinus*. However bean pods and pea pods highly significantly increased C_x activity. All other wastes slightly increased the activity of C_1 and C_x. The highest value of C_2 was obtained in cultures grown on wheat bran followed by bean pods. Cultures grown on both pea pods and sugarcane bagasse showed good production of C_2 without any significant differences.

Pea pods and cotton stem which stimulated the highest cellulolytic activities have been chosen to be added to wheat straw and rice straw (mainly used as substrates for mushroom production) at different concentrations to find out the best mixture concentration for enzymatic production.

TABLE 2. Effect of different wastes on production of cellulolytic activities by *P. columbinus*.

Wastes	Enzyme activities (units / ml)		
	C_1	C_x	C_2
Pea pods	0.78	0.25 c	0.56 a
Sugar cane bagasse	0.10	0.17 b	0.52 a
Cotton stem	0.21	0.21 b	0.35 b
Wheat bran	0.13	0.21 b	0.69
Rice straw	0.02 a	0.03 a	0.27 cb
Date – seeds	0.06 b	0.05 a	0.37 b
Corn stem	0.06 b	0.05 a	0.05 e
Wheat straw	0.03 ab	0.05 a	0.20 c
Rice husk	0.01 a	0.03 a	0.03 e
Sawdust	0	0	0
Bean pods	0.59	0.36	0.6

*** The legend as in Table 1.**

Tables 3 & 4 indicate that cultures of both *Pleurotus* spp grown on 3% cotton stem showed the highest cellulolytic activities. The concentration beyond 3% significantly decreased all enzymatic activities, however, concentrations lower than 3% were stimulatory to all enzymatic activities. Higher levels of cotton concentrations did not significantly appear to reduce C_1 & C_x activities compared with control (0.5% rice straw without addition of cotton stem). While 5% concentration of cotton stem significantly depressed C_1 and C_x activities by *P. pulmonarius* (Table 3). On the other hand, C_2 activity from cultures grown at both 4.5 and 5% cotton stem was significantly greater than control.

Unlike *P. pulmonarius* C_1 activity of *P. columbinus* was stimulated at higher concentrations of cotton stem. In addition C_2 activity from cultures grown at 4.5 & 5% were less than enzymatic activities from control, while C_x , as shown in *P. pulmonarius* was significantly depressed at higher concentrations of cotton stem (Table 4).

TABLE 3. Effect of addition different cotton stem concentrations to rice staw on cellulolytic activity production by *P. pulmonarius*.

Conc. %	Enzyme activities (units / ml		
	C_1	C_X	C_2
0	0.12b	0.12a	0.05
0.5	0.24	0.30e	0.18e
1	0.33a	0.33de	0.40d
1.5	0.68	0.76c	0.67a
2	0.76	0.78bc	0.68ab
2.5	0.90	0.82b	0.71cb
3	1.14	1.09	0.74
3.5	0.57	0.57	0.70c
4	0.36a	0.36d	0.38d
4.5	0.11b	0.11a	0.16e
5	0.06b	0.06	0.11

* The legend as in Table 1. 00.5% rice straw .

TABLE 4. Effect of addition of cotton stem concentrations to wheat staw on cellulolytic enzymes production by *P. columbinus*.

Conc. %	Enzyme activities (units / ml		
	C_1	C_X	C_2
0	0.21	0.24	0.46a
0.5	0.43a	0.61c	0.47a
1	0.69	0.72	0.48a
1.5	1.10	1.0	0.66
2	1.26	1.27	0.71b
2.5	1.39c	1.37b	0.72b
3	1.43c	1.41b	0.81
3.5	0.75	0.61c	0.78
4	0.68b	0.33	0.32
4.5	0.62b	0.14	0.28c
5	0.41a	0.04	0.26c

* The legend as in Table 1. 00.5% wheat straw .

Tables 5 & 6 show that all treatments had a strong response to pea pods levels, for both fungi in terms of cellulases activities, activity at 4% pea pods being significantly the greatest for all treatments. Unlike cotton stem, pea pods highly stimulated production of enzymes even at higher concentrations.The concentration 4% of pea pods was chosen for the optimization of Avicellase, since the highest enzymic activity has been achieved by both fungi.

TABLE 5. Effect of addition of pea pods concentrations to rice staw on cellulolytic production by *P. pulmonarius*.

Conc. %	Enzyme activities (units / ml (		
	C_1	C_X	C_2
0	0.14a	0.13b	0.22a
0.5	0.20a	0.27b	0.25
1	0.54b	0.77ec	0.39
1.5	0.59b	0.80ecd	0.42
2	0.71	1.23acd	0.51
2.5	1.15	1.27ad	0.61
3	1.23	1.66a	0.63
3.5	1.57	1.77	0.65
4	2.54	2.73	1.47
4.5	2.13	1.06edc	0.79
5	1.04	0.60be	0.21 a

*** The legend as in Table 1. 00.5% rice straw .**

TABLE 6. Effect of addition of pea pods concentrations to wheat staw on cellulolytic production by *P. columbinus*.

Conc. %	Enzyme activities (units / ml(		
	C_1	C_X	C_2
0	0.12	0.23	0.72
0.5	0.37	0.64a	0.82
1	0.57a	0.66a	0.92a
1.5	0.65	0.97b	1.01a
2	1.07c	1.15bc	1.11
2.5	1.50	1.28c	1.27
3	1.65	1.46	2.86
3.5	1.97	3.18	4.82
4	2.06	3.40	6.77
4.5	1.13c	2.15	2.94
5	0.54a	1.92	1.51

*** The legend as in Table 1. 00.5% wheat straw .**

Figure 1 exhibits that the maximum production of Avicellase was achieved on the seventh and eighth days of growth by both *P. pulmonarius* and *P. columbinus*. . Further incubation time decreased the rate of enzyme production.

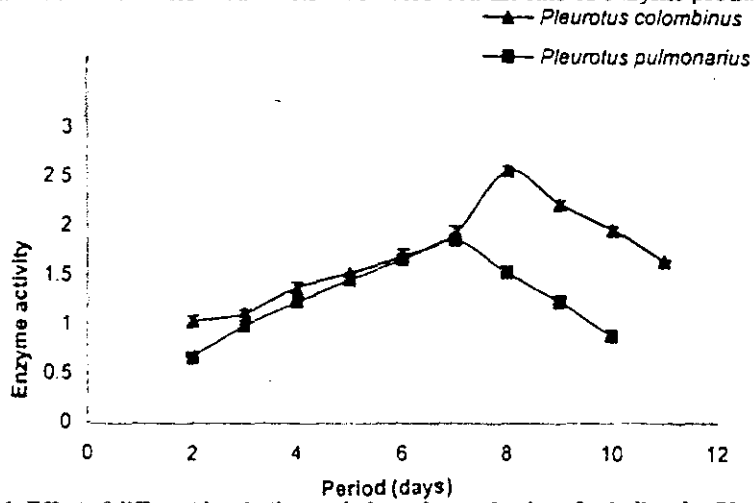

Fig.1. Effect of different incubation periods on the production of avicellase by *Pleurotus* spp. Data represented as means of three replicates with standard errors.

Figure 2 illustrates an optimal pH plateau for Avicellase activity of both *Pleurotus* spp. It exists from pH 5 to 7 for *P. columbinus* and from pH 5 to 6.5 for *P. pulmonarius*. pH 5.5 gave the highest activity for both *Pleurotus* spp therefore, this pH was used for enzyme assay. It is obvious from Fig. 2 that activity of C_1 of *P. columbinus* was significantly higher in alkalinity than in acidity shifts, although the reverse results could be noticed in *P. pulmonarius*.

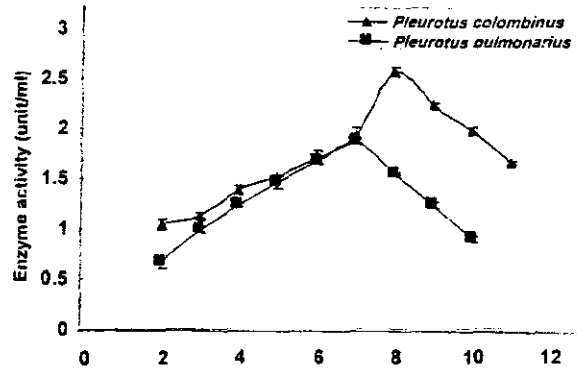

Fig.2. Effect of initial pH values on the production of avicellase by *Pleurotus* spp. Data represented as means of three replicates with standard errors.

Asparagine and potassium nitrate significantly stimulated C_1 activity by *P. columbinus* and *P.pulmonarius* respectively. Ammonium chloride and ammonium nitrate stimulated good C_1 activity by both *Pleurotus* spp. (Fig. 3). Increasing nitrogen concentration stimulated Avicallase activity of both fungi up to 3.2 gN/l (Fig. 4).

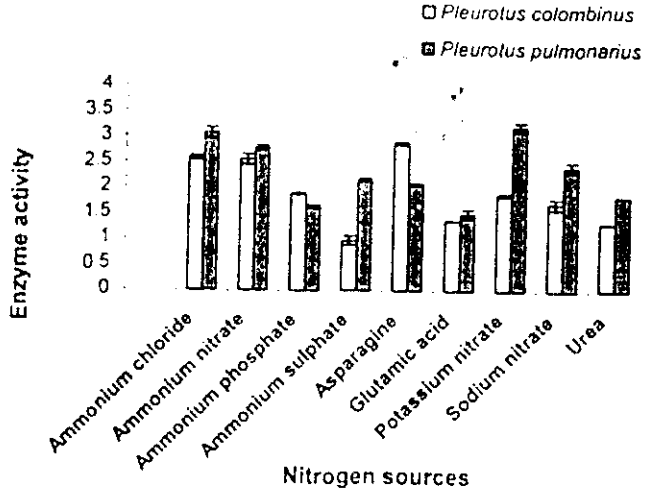

Fig.3. Avicellase content of *Pleurotus* spp. as influenced by different nitrogen sources. Data represented as means of three replicates with standard errors.

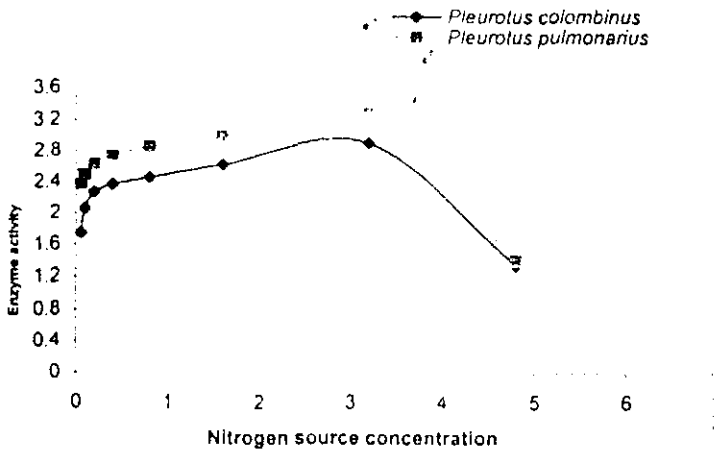

Fig.4. Behaviour of *Pleurotus* spp. In Avicellase production as a function of different concentrations of nitrogen source. Data represented as means of three replicates with standard errors.

Discussion

One of the most economically viable processes for the bioconversion of many types of lignocellulosic wastes is represented by edible mushroom cultivation. The *Pleurotus* is one of the important commercially cultivated mushrooms which exhibits varying abilities to utilise different lignocellulosics as growth substances (Buswell *et al.*, 1996). High cellulolytic activity has been achieved by both tested species grown on pea and broad bean pods, wheat bran, cotton stems and sugarcane bagasse. In addition, it had been reported that lignocellulolytic activity of *P. pulmonarius* in liquid culture was enhanced by the presence of lignocellulosic substrates such as mixture of cotton stem and wheat straws (Masaphy and Levanon, 1992).

Recent improvement of cellulase production involved the use of high level cellulose as substrate (Dhillon *et al.*, 1988 and Nour El-Dein, 1992). Bean pods, pea pods and wheat bran stimulated cellulolytic activities of both *Pleurotus spp.* The stimulatory effect of wheat bran has been reported by Xiao-Bin *et al.* (1998) to increase cellulases by *Trichoderma reesei.* Nour El-Dein (1992) found that high growth and cellulolytic activities of all tested *Aspergillus spp* and *Fusarium spp* were obtained when the fungi were grown on bean pods, pea pods and wheat bran. Those wastes contain higher nitrogen contents than the other tested wastes (Nour El-Dein, 1992). Accordingly, there is an increased need for fast turnover of the proteins to enzymes capable of degrading the polymers introduced into the medium with additives, and increase enzyme production. This explanation is supported by the higher enzymatic activities achieved in these media. Most studies had also shown that basidiomycetes respond best to higher nitrogen for celluloysis (Reid, 1983 and El-Fallal, 1990). Also, Bisaria *et al.* (1987) and Hassan (1992) concluded that wheat bran increased the nitrogen content in fruit bodies of *Pleurotus* spp.

However, it was reported that *P. ostreatus* grown and yielded well on wheat straw, biosynthesising active cellulase complexes, glucohydrolases, both endo- and exo-gluocanase and β-glucosidase (Chrapkowska and Podyma, 2000). The present study came to the same conclusions, although lower enzymic activities were obtained either in wheat straw or rice straw.

P. pulmonarius reacted positively to the addition of cotton wheat straw (CWS) when grown in liquid culture. The presence of CWS enhanced lignocellulolytic activity and the rate of consumption of soluble carbon and nitrogen (Masaphy and Levanon, 1992). The stimulatory effect of cotton stem was ascribed to the presence of growth promoter in the soluble fraction of cotton stem (Platt *et al.* 1984). Also El-Fallal and El-Kattan (1997) recorded that a water extract of cotton stem stimulated the mycelial growth of *Pleurotus floridanus*, *Voloariella volvacea* and *Agaricus bisporus*. Furthermore, the addition of the cotton stem to wheat straw increase the biological efficiency of *P. floridanus* and its degradation of lignocellulose (El-Fallal, 1995).

Studies on the optimization of the cellulolytic enzymes production revealed that the maximum production of Avicellase by *P. columbinus* and *P. pulmonarius* was after 7 and 8 days incubation. The maximal exo β 1,4-glucanase activity of *V. volvacea* was obtained within 5 days. The degradation of cellulose by *sajor caju* was rapid at initial stages of growth. The activity of exoglucanas was maximum at 12 days of growth (Kannan and Oblisami, 1990). It has been recorded that cellulase production by *Trichoderma koningii* reached its maximal after 6 days of incubation (Kassim, 1983). Moreover, Dhillon *et al.(*1988) obtained the highest yield of *T. reesei* cellulases after 7 days of incubation on rice straw-containing medium.

The optimal pH for the Avicellase activity in the present work was 5.5 and 6 for *P. columbinus* and *P. pulmonarius* respectively. It was found that optimal pH for cellulase activity by *V.volvacea* was 4.8-5 (Chang and Steinkraus, 1982).With the initial pH value of 6.5 *Neurospora crassa* yielded the higher activities for all cellulolytic enzymes on milled wheat straw (Romero *et al.*, 1999). Optimal pH for activity of cellulase system by *Nectria catalinensis* was registered between 4.2-5.8 (Pardo and Forchiassin, 1999). Moreover pH 4.5-5 and 5 were the optima for cellulases by *Trichoderma longibrachiatum* and *T. koningii* respectively (Sandhu and Kalra, 1985) and (Kassim, 1983).

In the present work, asparagine and potassium nitrate were the best nitrogen sources for Avicellase production by *P.columbinus* and *P.pulmonarius respectively. Asparagine* at 1gN/l could be considered the best nitrogen source for cellulose utilization for all basidiomycetes tested including *P. floridanus*, except for *Coprinus cinereus* which showed a strong response to inorganic nitrogen, both as ammonium and nitrate (El-Fallal, 1990). Organic nitrogen seemed to promote optimal cellulolysis. Most other studies had also shown that basidiomycetes respond best to organic nitrogen (Reid, 1983). However *Coriolus versicolor, L. edodes* and *P. floridanus* showed a response to ammonium nitrogen (El-Fallal, 1990). This study agree with the present investigation, since cellulolysis of both *Pleurotus* species responded well to ammonium salts. Additionally, inorganic sources of nitrogen seemed to be more suitable for different cellulolytic activity by *Aspergillus sydowii* and *Fusarium accuminatum* (Nour El-Dein, 1992). It is worthy to state that *P. pulmonarius* appeared to utilise asparagine best for its growth (El-Fallal *et al.* in press), but appeared not to be preferred in cellulolysis.

King and Smith (1973) reported that growth and cellulolytic enzyme production were similar with asparagine and with urea. They added that growth of *Coniophora* in ammonium sulphate was very poor and little enzyme was produced. The present study came to the same conclusion only with *P. colombinus*. However urea neither supports growth (El-Fallal *et al.* in press) nor cellulolysis by both tested fungi.

Increasing nitrogen levels stimulated cellulolytic activity in this study. Also El-Fallal (1990) working on *V. volvacea*, found with increasing nitrogen levels, cellulolysis increased. King and Smith (1973) recorded that cultures of *Coniophora* with highest levels of asparagine were significantly better in cellulolytic activity than with lower levels.

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إنتاج الانزيمات المحللة للسليلوز بواسطة نوعين من فطرة بليوروتس النامية على بعض المخلفات الزراعية

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تم قياس مقدرة الفطرتين بليوروتس بالمونيرس و بليـوروتس كـولمبينس علـى انتـاج إنزيمات السليوليز فى وجود بعض النفايات الزراعية. ووجد أن قرون البسـلة وسـيقان القطن هى أفضل النفايات المستخدمة فى الانتـاج بواسطة الفطرتين عندما أضيفت الى المنبت السـائل كمصدر كـربونى .

و أتضح كذلك أن قرون الفول و مصاصة القصب تدعم الانتـاج العـالى للفطـرة بليورتس بالمونيرس و بليوروتس كولمبينس على التوالى ، وبإضافة قرون البسلة وسـاق القطن منفصلة الى الوسط السائل المحتوى على قش القمح أو قش الارز بتركيزات ٤٪ ، ٣٪ على التوالى تؤكد زاد إنتـاج الانزيمات بالفطرتين .

و قد تبين أن C_1 يعطى أقصى إنتاج و لذا تم اختياره لتعيين أمثل الظروف للإنتاج و كانت أفضل فترة تحضين بعد اليوم السابع و الثامن و رقم أس هيدروجينى ٥ ، ٥ ، ٦ و أن نترات البوتاسيوم و الأسباراجين هى أحسن المصادر النيتروجينية للفطرتين بليوروتس بالمونيرس و بليوروتس كولمبينس على التوالى .