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PRODUCTION OF ETHANOL BY *TRICHODERMA* SPP IN SOLIDSTATE FERMENTATION OF SUGARCANE MOLACESS.

NANDITA JENA[1]

S. SATPATHY[2]

IASE University (Off Campus), Mancheswar Industrial Estate, Bhubaneswar, INDIA.

Institute of Agricultural Sciences, SOA University, Bhubaneswar-751030, ODISHA, INDIA.

Abstract

Production of ethanol from molasses fermentation by different microbes was studied in a sugar factory. *Sacharymyces* (Yeast) and *Trichoderma* spp. are the predominant organisms contributed 24.44 and 35.59 percent population contribution. *Trichoderma* spp. can produce highest quantity of ethanol (0.52 ml/gm) when glucose is used as substrate followed by cellulose and xylose. Highest 0.37 ml/gm ethanol is produced during molasses fermentation by *Trichoderma* followed by *Sacharomyses*. Commercial ethanol production hence may be recommended from molasses using *Trchoderma* spp.

INTRODUCTION

Agro-biotechnology is basically an industry-based-process which deals with production of foods, and the processing of agricultural waste products. They could yield commercially valuable products by the action of specific micro-organisms. Industrial process using micro-organisms exploit the enzymatic activities of the microbes to produce substances of commercial value. This technology utilized in fermentation to obtain maximum growth of an organism under the optimum physical conditions in a specific medium for the production of desired end products. In the view of practical and commercial values of micro-organisms, several problems are associated with commercial exploitation of micro organisms. The most important considerations involved in any microbial process are the selection of suitable substrate. The industrial process is more economical and commercial when one high yielding strain is being developed.

Production of ethanol from biomass has been considered as a goal because plant biomass is the only sustainable source of organic fuels and chemicals. In most of the process, cellulose biomass is first enzymatically hydrolyzed to sugars, often with fungal celluloses and then transform to ethanol. To lower the cost associated with this pre-treatment, it is desirable to accomplish the fermentation of cellulose to ethanol in one step. In this consolidated bio-processing or direct microbial conversion are carried out by one micro-organism or a consortium of micro-organisms in a single bioreactor (Lynd et al 1999). Anaerobic bacteria are the potential organisms to convert cellulose biomass to ethanol. However, some filamentous fungi hold promise in this area and there are some advantages to them as fungi are the organisms that do not require strictly anaerobic conditions and so can be directly inoculated into cellulose biomass and their filamentous habit facilitates separation of cell mass from the broth. Many fungal strains produce copious numbers of conidiophores, which could be useful for inoculation at a high level, making the inoculation of non-sterile biomass more practical. Several filamentous fungi have been reported which directly ferment cellulose to ethanol. These include members of the genera *Aspergillus, Rhizopus* (S Kory et al. 1997), *Monilia* (Gong et al. 1981), *Neurospora* (Despande et al 1986), *Fusarium* and *Trichoderma* (Prasad et al. 2013).

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In this study, environmental samples were screened for organisms capable of producing ethanol from cellulose on minimal medium. We have isolated a filamentous fungus that capable of this conversion and have made some progress towards increased ethanol yields and development of a genetic system based on Para sexuality (Bagagli et al 1995) from celluloid wastes extracted from sugar cane based industries.

MATERIALS AND METHOD

Culture media:

The suitable media used in this study were Potato Dextrose Agar (PDA), Yeast Peptone Malt extract (YPM), and minimal media (M.M). PDA was prepared by taking 200 gm of peeled potatoes, 20 gm of dextrose and 15 gm of Agar. YPM was prepared by taking yeast extract 2 gm; peptone 2 gm and malt extract 5 gm (S Kory, et al, 1997). Minimal Medium (MM) was the yeast nitrogen base medium of Wickerham and Burton (1948), without added vitamins and was adjusted the pH to 5.0.

Molasses Composition:

Molasses is the waste product of sugar factory. Molasses was collected from Sakti Sugar Limited, Korian, Haripur, Dhenkanal district of Orissa state, India to carry out the study under this project. The fresh as well as the solid state fermented molasses were collected in sterilized glass containers with lid for the study. The compositions of molasses were studied. Total sugar (reducing and non-reducing), cellulose, nitrogen, sulpher, ash and moisture content of the molasses were analyzed by standard procedures (Mahadevan and Sridhar, 1996).

Isolation of Micro Organisms:

Micro organisms associated with solid state fermentation of molasses were isolated from formatted molasses waste of sugar factory situated at Dhenkanal of Orissa state. The micro organisms were isolated in PDA (Potato Dextrose Agar), Yeast Peptone Malt extract (YPM) as described by Skory et al 1997. Minimal Medium (MM) which was the yeast nitrogen base medium without vitamins (Wickerham and Burton, 1948). The fungal, bacterial and yeast species were isolated by serial dilution and streaking methods on agar supplemented media plates. The microorganisms were made pure and grown in culture tubes with growth media without any vitamins and growth regulators. The percentage contributions of different organisms were calculated.

Inoculums Preparation:

Sugar cane molasses was mixed with mineral salt solution 3:5:1 (w/w) and pH was adjusted to 5.0 with sulphuric acid. This mixture was then sterilized it 115˚C for 60 minutes in an autoclave. The spore suspension of the micro organism, isolated separately inoculated to the mixture and incubated at 28 ±2˚C in an incubator for inoculums production. To check for contamination it was periodically observed by phase contrast microscope and streaked on to common bacterial growth media LB agar.

Study of morphological/ physiological behavior of *Trichoderma*

Trichoderma is filamentous fungi found to grow rapidly and when mature it produced dark green conidia on minimal medium (Stevenson and Weimer 2002). Its morphology suggests that, it is a member of the section Trichoderma of genus *Trichoderma* (Bissett 1991, Gams and Bissett, 1998), although its identification based on morphology. The species of *Trichoderma* where found to be similar but not identical (Samuels, 1996). Its optimal growth temperature was determined by inoculating 5 ml of YPM with conidia and incubating without shaking at

various temperatures. The mycelia mass was collected after 94 hours of inoculation and the dry weight was taken. Similarly the optimum pH was also determined.

Fermentation

Anaerobic fermentation of sugar, cellulose along with other substrates of molasses was studied using two growth methods, sealed flask method and vented flask method. In the sealed flask method 200 ml of sterile molasses medium in 250 ml Erlenmeyer flask was simply inoculated with conidia and the culture vessel was sealed with either a butyl stopper or screw-cap. Thus approximately 20% of the culture flask volume was left as air space. The oxygen in this space allowed mycelia to develop before the flask become anaerobic and fermentation began. In the second (vented flask) method the culture flask was prepared as in sealed flask method but the stopper used to seal the flask was vented through an inserted 26 gauze needle capped with a 3 ml syringe barrel packed tightly with cotton (Stevenson and Weimer 2002).

Then the supernatant was aseptically decanted away from the dense, stable mat and the formulation products were analyzed in culture supernatant by HPCL (Weimer et al. 1991).

RESULTS

Composition of molasses:

Molasses is consist of approximately 50% carbohydrate (reducing and non-reducing), 11% cellulose, 9% ash and 28% moisture content. Besides these, very little amount of nitrogen, sulphur and volatile acidic substance and protein were also found to be present. The constituents are presented in Table 1.

Isolation of microbes:

The microbial association with solid state fermentation of molasses was studied in PDA mediated petriplates. The organisms isolated were *Sacharomyces* (Yeast), *Bacillus* and the fungal members like *Penicillumn* sp. *Aspergillus* sp. *Trichoderma* sp. and *Mucor* sp. There percentage population contribution is presented in Table 2. *Trichoderma* contributed highest 35.59% to the total population followed by yeast 24.44%. *Penicillium* and *Humicola* contributed very low (4.44%) population to the total (Table 2).

Physiological behavior of *Trichoderma* sp.

To find out the optimal temperature and pH for growth of the *Trichoderma* sp. one experiment was conducted and the result is presented in Table 3 & 4. The optimum temperature for its growth was found to be 32˚C and the maximum growth was at 4.0 pH level. *Trichoderma* found to be growing better in slight acidic medium. Thought the optimum temperature was 32˚ but it shows better growth within the temperature range of 25˚ and 38˚C. The pH range was 3.5 to 4.5 for its better growth (Table 4).

Molasses fermentation by different microorganisms:

Production of ethanol by different microbes were studied separately in three rounds are presented in Table 5. From the results it is revealed that *Trichoderma* sp. was able to produce ethanol in total maximum 0.37 ml per gram fresh molasses followed by *Saccharomyces* (yeast) 0.32 ml/gm. Lowest production of ethanol was noted by *Hemicala* sp. (0.08 ml gm^{-1}). The other microorganisms like species of *Bacillus*, *Pencillium, Aspergillus* and *Mucor* sp. produces total 0.18, 0.16, 0.12 and 0.09 ml gm^{-1} of fresh molasses. *Trichoderma* sp. produce comparatively more quantity of ethanol in the second round (Table 5).

Ethanol production in different carbon sources

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The production of ethanol by *Trichoderma* sp. was tested using different carbon sources. When glucose was used alone, *Trichoderma* is able to produce 0.52 ml/gm followed by cellulose 0.21 ml gm^{-1}. But ethanol production was very low i.e. 0.07 ml/gm when xylose was used as the substrate (Table 6).

DISCUSSION AND CONCLUSION

The brewing industry and the wine industries are dependent upon alcohol producing capability of yeast and some other important products are also obtained by using microbes. The petroleum industry uses microorganisms as indicators in exploring for new reserves.

But in recent years, cellulose also have become a district possibility as a renewable source of alcohol. Bagasse sulphite waste, liquor of paper industry and agricultural biomass are the sources of cellulose. Direct fermentation of cellulose to ethanol is of current interest. Besides the unicellular fungus, yeast, other filamentous, fungus like *Aspergillus, Penicullium, Neurospra, Mucor, Monillia, Trichoderma* etc. are also carry on ethanol production.

Molasses is the waste product or the drained off materials of sugar producing factory which is enriched with adequate amount of carbohydrates (more than 50%) cellulose, ash and moisture. Besides these negligible amount of nitrogen, sulphur and volatile acid substance are also present (Gupta, 1999). Ethanol is generally produced by fermentation of sugar rich products with the help of yeast (Saccharomyces). Beside this some other organisms like fungi and bacteria also produce small quantity of ethanol. But in later stages works on solid state fermentation confirmed that the class of micro organisms that are most commonly used is Fungi in bio processing (Zheng and Shetty, 2000; Pandey et al. 2000). Ethanol production by filamentous fungi and anaerobic conditions is relatively unusual (Stevenson and Weimer, 2002). Several agro-industrial waste and bi-products such as orange bagasse (Martins et al 2002, Sugar Cane bagasse (Silva et al 2002, Pandey et al., 2000, Prasad et al. 2013) wheat bran (Cavalitto et al 1996) and other food processing waste (Zheng and Shetty 2000) are effective substrates for ethanol and enzyme production. Recently *Trichoderma* strain is proved to be capable of fermenting cellulose to ethanol (Stevenson and Weimer 2002). Other fungi like *Penicillium, Aspergillus, Humicola* and *Mucor* species are also able to produce ethanol but in less amount. Therefore commercial ethanol production is not suitable by these fungi (Grajek 1987). But *Trichoderma* may be used in commercial production of ethanol as it is capable to break the cellulose to reducing sugar which in later stage converted to ethanol in anaerobic condition.

Several characteristics of *Trichoderma* which was isolated from solid state fermented molasses are desirable in an organism used for the biological production of ethanol are : (1) aerobic growth and anaerobic fermentation both occurred in minimal media, (2) the low pH which this organisms favors (Table 4) reduces the potential for contamination by ethanol utilizing bacteria, and (3) the organism is enable to genetic manipulation via parasexuality. The lack of a genetic system has been a major impediment to the use of several species of bacteria for consolidated bio-processing (CBP) (Lynd et al. 1999). A number of mutant *Trichoderma* strains are available with all the three characters (Toyama et al. 1984, Stasz et al. 1989, Stasz and Harman, 1990).

During the course of this study ethanol yield from molasses by *Trichoderma* significantly increased. Perhaps the most important factor in the increase of ethanol yield was the use of vented culture as opposed to strictly anaerobic incubation. The mechanism of this improvement is not clear, but it could have been due to the additional O_2 (Oxygen) available during the initial growth and generation of Carbon dioxide (Co_2) in the later stage.

Increasing biomass density along played little role in increasing ethanol production. *Trichoderma* is able to produces more ethanol from reducing sugar i.e. glucose in first round and then the rate decreased in second

and third round. But when cellulose is used as substrate ethanol production was less in 1st round and increased in second round. This may be due to the breakdown of cellulose 1st to reducing sugar which is converted to ethanol in the later stage.

Trichoderma is capable of utilizing numerous compounds as carbon sources when the organism was grown aerobically. This wide range exceeds that described for several *Trichoderma* strains (Manczinger and Polner 1987), In contrast to glucose and cellulose, very little amount of ethanol was formed when xylose was the substrate (Table 6), although other fermentation products were formed. Many of the co-products included the TCA cycle intermediates as well as several unidentified compounds. As the molasses is composed of reducing sugar (50%) and cellulose of 11% it is seems to be the ethanol production is more by *Trichoderma* due to the combine action of the organism on both the substrates like glucose and cellulose. But in other hand organisms that are used for ethanol production like Sachharomyces (yeast) and Bacillus are not able to efficiently break down the cellulose to glucose; hence the ethanol production is reduced.

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Table 1 : Chemical composition of Molasses sample under study.

Sl. No.	Component	Composition (%)
1.	Total carbohydrates (reducing and non-reducing) Fermentable 45% Non-fermentable 5%	50.00
2.	Cellulose	11.00
3.	Protein	1.30
4.	Total nitrogen	0.40
5.	Volatile acid substance	0.10
6.	Total sulphur	0.20
7.	Ash	9.00
8.	Moisture	28.00

Table 2 : Percentage contribution of microorganisms associated with solid state molasses fermentation .

Sl. No.	Microorganisms	Population (% contribution
1.	*Saccharymyces (yeast)*	24.44
2.	*Bacillus*	15.55
3.	*Pencillium sp.*	4.44
4.	*Aspergillus sp.*	6.66
5.	*Helmicola sp.*	4.44
6.	*Mucor sp.*	8.88
7.	*Trichoderma sp.*	35.59

Table 3. Growth of *Trichoderma* at different temperature

Temperature (°C)	Colony Diameter (mm)			Dry mycelia wt. at 15 days mg.
	3rd day	5th day	7th day	
20	10.66	20.33	38.66	380.00
25	11.66	23.00	49.00	463.33
30	13.33	29.33	56.00	543.00
32	18.33	38.66	69.66	578.33
35	15.00	31.33	60.00	528.00
38	14.33	28.66	55.33	499.33
40	11.33	25.66	48.66	388.66
45	8.33	15.33	28.33	138.00

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Table 4. Growth of *Trichoderma* at different $P_{H.}$

pH Range			
Before sterilization	After sterilization	Dry mycelia weight (mg)	
2.1	4.0	129.0	62.38
2.5	4.5	238.0	15.63
3.0	5.0	428.3	108.18
3.5	5.5	452.6	59.29
4.0	5.9	520.3	104.4
4.5	6.4	518.0	53.32
5.0	7.4	514.6	48.63
CD P = 0.05		7.60	

Table 5. Ethanol production in Molasses formation by microorganisms (ml/gm of molasses).

Microorganisms	Ethanol production (ml/gm)			Total
	1st round	2nd round	3rd round	
1. *Sacharomyces* (A1)	0.22	0.06	0.04	0.32
2. *Basillus* (A2)	0.13	0.04	0.01	0.18
3. *Penicullium* sp. (A3)	0.09	0.04	0.03	0.16
4. *Aspergillus* sp. (A4)	0.08	0.04	0.00	0.12
5. *Hamicola* sp. (A5)	0.05	0.02	0.01	0.08
6. *Mucor* sp. (A6)	0.06	0.03	0.00	0.09
7. *Trichoderma* sp. (A7)	0.18	0.11	0.08	0.37

Table 6. Ethanol production in different carbon sources by *Trichoderma* sp.

Carbon source	Ethanol production (ml/gm)			Total
	1st round	2nd round	3rd round	
1. Glucose	0.25	0.18	0.09	0.52
2. Xylose	0.02	0.03	0.02	0.07
3. Cellulose	0.05	0.13	0.03	0.21