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# Effect of substrate concentration and cell loading on the hydrolysis of β(1-4) glycosidic bond in orange mesocarp (*Citrus sinensis*) by trichoderma reesei for glucose production

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**ABSTRACT**

*In this study, we evaluate Orange mesocarp as a feed stock for production of glucose syrup using Trichoderma. reesei. The material was crushed to 100 – 150* µm*, 200 – 250* µm *and 300 – 425µm particle sizes and fed into a bioreactor where delignification was effected.A series of enzymatic hydrolysis was carried out at different substrate concentrations of 0.2g/L, 0.4g/L, 0.6g/L, 0.8g/L, 1.0g/L, 1.2g/L1.4g/L, 1.6g/L, 1.8g/L and 2.0g/L, while other reaction conditions were kept constant. Additional series of enzymatic hydrolysis was carried out at different cell concentration of 0.01gL$^{-1}$, 0.02gL$^{-1}$, 0.03gL$^{-1}$, 0.04gL$^{-1}$, 0.05gL$^{-1}$, 0.06gL$^{-1}$, 0.07gL$^{-1}$, 0.08gL$^{-1}$, 0.09gL$^{-1}$ and 0.10gL$^{-1}$. In separate runs, the effect of substrate concentration and cell loading on extent of hydrolysis was studied. Our results reveal that as the substrate concentration increases from 0.2gl$^{-1}$ to 2.0gl$^{-1}$, there was a corresponding increase in glucose concentration from 1.2mmolL$^{-1}$ to 2.13mmolL-1 and 0.4mmolL$^{-1}$ to 2.13mmol$^{-1}$ at Cs = 0.6g and 1.2g respectively as time increases. Similarly, there was a substantial increase in glucose concentration with increase in cell loading.*

**Key words**: Orange Mesocarp, Glucose, *Trichoderma reesei*

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## INTRODUCTION

Orange is botanically a family whose dominant members include sweet orange (citrus sinesis), tangerine orange (citrus reticulate), grape fruit (citrus paradisi). Sweet orange (citrus sinesis) production in Nigeria is significant, with heavy direct consumption due primarily to a few and small capacity processing industries to convert the fruit to juice, concentrate and canned fruit [1,2,3].

Orange mesocarp is an agricultural waste material capable of removing toxic heavy metals from aqueous solution by absorption, chelation and ion exchange [4]. The exchange properties of these agricultural wastes can be attributed to the presence of carboxylic phenolic, hydroxylic, cyano groups etc. These functional groups attract and sequester metal ion [5].Orange mesocarp contains various carbohydrate polymers. Particularly, its cellulose content ranges between 30-60% [6,7] which makes it an interesting choice for production of metabolites such as fermentable sugars and ethanol by appropriate micro-organisms.

The naturally high degree of crysallinity due to the tightly packed crystallites in the cellulose in the orange mesocarp, causes entanglement of the lignin and hemicelluloses in the cellulose matrix; thus leading to poor glucose

yield. Pre-treatment is however required to break down the cellulosic complex structure to its simpler components i.e. cellulose, hemicellulose and lignin polymers, prior to their conversion to the sugar monomers [6]. It has been discovered that proper treatment of cellulose can change them from liabilities to assets [8].

*Trichoderma reesei* is a mesophilic and filamentous fungus. It is an anamorph of the fungus *Hypocrea jecorina.T. reesei* has the capacity to secret large amount of cellulolytic enzymes (cellulases and hemicellulases). Microbial cellulases have industrial application in the conversion of cellulose, a major component of plant biomass, to glucose. Recent advancement in the biochemistry of cellulose enzymology: the mechanism of cellulose hydrolysis, strain improvement, molecular cloning and process engineering are bringing *T. reesei* cellulases closer to being a commercially viable route to cellulose hydrolysis. Major advances have been made in the isolation of Trichoderm mutants [9].

Novozyme reported studies on enzymatic hydrolysis of cellulose using *Trichoderma reesei* cellulase. Also enzyme and chelating agents in cotton pretreatment has been reported by Emilia *et al*.,(2001). Waag et al., (2005) studied the efficient cellulose production from corn straw by *Trichoderma reseei*. Little information, however, exist in literature concerning the effect of substrate concentration and cell loading on hydrolysis of glycosidic bond of orange mesocarp. It is therefore the goal of this research to study the conversion of a chemically treated orange mesocarp to glucose using cell of *Trichoderm reesei*.

## MATERIALS AND METHODS

Orange mesocarp were collected, ground into particle sizes ($P_1$, $P_2$, $P_3$). These particle sizes were pretreated by three distinct pretreatment agents (SAC); sodium hydroxide, ammonia and calcium hydroxide. Pretreatment breaks down the lignin, allowing access to the cellulose and hemicelluloses. This increases porosity and reduces cellulose fibre crystalinity (tightly packed crystallites)[9]. The hemicelluloses and cellulose are also separated from each other allowing for more efficient access by hydrolysis enzyme. Applying the method used by Yakubu *et al*., (2001), 4g of orange mesocarp (OMP) was weighed, and pre-treated at varying pretreatment conditions (0.1M, 0.2M, 0.3M and 0.4M) Sodium hydroxide (NaOH) and calcium hydroxide $Ca(OH)_2$ at $100^oC$ and time (15, 20, 25 and 30mins) in different runs.

Additionally, the ammonia steeping method too was employed to delignify the orange mesocarp. The delignified mesocarp was treated with 0.3M HCl acid at $100^0C$ for 1 hour to remove hemicelluloses. The pre-treated cellulose was washed with de – ionized water to remove residual acid. All samples were dried in an oven at $50^oC$ for 48 hours and kept in the laboratory stock for further use.

In a typical run, the temperature of the water bath (sharmond model) was set at $37^0c$, a hundred milliliters (100ml) of 0.1M sodium acetate buffer solution (pH 4.5) was poured into an Erlenmeyer flask fitted with stimrig mechanism. 0.1g of isolated cellulose enzyme and 2.0g of pre-treated orange mesocarp of different particle sizes were added. 40 micro litres of each sample were withdrawn every 4hrs within 58 – 72hrs reaction time for analysis. The glucose concentration in the sample was determined by using Randox glucose kit and colorimeter (Model WPA, 5001, USA) at 540nm[7,10]. Each run was repeated three times and the average was taken to assume accuracy. In separate runs, the effect of substrate concentration and cell loading on extent of hydrolysis was studied.

A series of enzymatic hydrolysis was carried out at different substrate concentrations of 0.2g/L, 0.4g/L, 0.6g/L, 0.8g/L, 1.0g/L, 1.2g/L1.4g/L, 1.6g/L, 1.8g/L and 2.0g/L, while other reaction conditions were kept constant.

Additional series of enzymatic hydrolysis was carried out at different cell concentration of $0.01gL^{-1}$, $0.02gL^{-1}$, $0.03gL^{-1}$, $0.04gL^{-1}$, $0.05gL^{-1}$, $0.06gL^{-1}$, $0.07gL^{-1}$, $0.08gL^{-1}$, $0.09gL^{-1}$ and $0.10gL^{-1}$.

Other conditions were identical to the normal hydrolysis condition. Then the results were analyzed for glucose concentration produced.

## RESULTS AND DISCUSSION

### Effects of Substrate Concentration

The results are presented in Table 1.From the results of effect of substrate concentration, it is deduced that as the substrate concentration increases from 0.2gl$^{-1}$ to 2.0gl$^{-1}$, there was a corresponding increase in glucose concentration from 1.2mmolL$^{-1}$ to 2.13mmolL-1 and 0.4mmolL$^{-1}$ to 2.13mmol$^{-1}$ at Cs = 0.6g and 1.2g respectively as time increases. This trend is not unusual because similar results were reported on the hydrolysis of rice straw using *Aspergilus niger*, animal manure, soft wood, weeds and bagasse [4,11]. This can be further explained from the point of view that, increasing the substrate concentration without a corresponding increase in enzyme concentration amounts to availability of more cellulose in the bioreactor for hydrolysis.

**Table 1: Effect of substrate concentration (g/l)**

| Glucose concentration (mmol/L) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| T(h) | 0.20 | 0.40 | 0.60 | 0.80 | 1.00 | 1.20 | 1.40 | 1.60 | 1.80 | 2.00 |
| **Substrate Concentration (g/l)** | | | | | | | | | | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 1.7072 | 1.4938 | 1.2804 | 0.8536 | 1.4938 | 0.4268 | 0.2134 | 0.2134 | 0.2134 | 0.2134 |
| 8 | 1.9206 | 1.4938 | 1.9206 | 1.4938 | 1.7072 | 0.8536 | 1.4938 | 1.4938 | 1.067 | 1.067 |
| 10 | 1.9206 | 1.7072 | 1.9206 | 1.7072 | 1.7072 | 1.067 | 1.4938 | 1.4938 | 1.067 | 1.067 |
| 24 | 1.9206 | 1.9206 | 2.134 | 1.9206 | 1.9206 | 2.134 | 1.9206 | 1.7072 | 2.134 | 1.7072 |
| 28 | 1.9206 | 1.9206 | 2.134 | 1.9206 | 1.9206 | 2.134 | 1.9206 | 1.9206 | 2.134 | 1.9206 |
| 30 | 1.7076 | 1.9206 | 2.134 | 1.9206 | 1.9206 | 2.134 | 1.9206 | 1.9206 | 2.134 | 2.134 |
| 34 | 1.7076 | 1.7072 | 2.134 | 1.7072 | 1.9206 | 2.134 | 1.9206 | 2.134 | 2.134 | 2.134 |
| 48 | 1.7076 | 1.7072 | 2.134 | 1.7072 | 1.9206 | 1.7072 | 1.9206 | 2.134 | 1.7072 | 2.134 |
| 52 | 1.7076 | 1.7072 | 2.134 | 1.7072 | 1.9206 | 1.7072 | 1.9206 | 2.134 | 1.7072 | 2.134 |
| 58 | 1.7076 | 1.7072 | 2.134 | 1.7072 | 1.9206 | 1.7072 | 1.9206 | 2.134 | 1.7072 | 2.134 |
| 72 | 1.7076 | 1.7072 | 2.134 | 1.7072 | 1.9206 | 1.7072 | 1.9206 | 2.134 | 1.7072 | 2.134 |

*Substrate ≤ 2g/l*
*Enzyme = 0.1g/l*
*Temp = 37$^0$C*
*pH = 4.5*

Thus, the activity of endoglucanase and cellobiohydrolase will be reduced since there is more cellulose for it to hydrolyse. Also, there will be sugar depletion from the substrate into the medium and possible use of glucose by fungi to supply its internal energy (metabolic) requirement [9].

**Table 2: Effect of Cell Loading (g/l)**

| Glucose concentration (mmol/L) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cell load/g/L | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | 0.09 | 0.1 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 1.7072 | 1.067 | 1.9206 | 1.7072 | 1.2804 | 2.134 | 1.4938 | 1.2804 | 1.4938 | 1.4938 |
| 8 | 1.9206 | 1.4934 | 2.134 | 1.9206 | 1.4938 | 2.134 | 1.7072 | 1.4938 | 1.7072 | 1.7072 |
| 10 | 2.3474 | 2.7742 | 2.5608 | 1.4938 | 1.4938 | 1.9206 | 1.7072 | 1.4938 | 2.3474 | 1.7202 |
| 24 | 2.3474 | 1.9206 | 1.7072 | 1.7072 | 1.7072 | 1.7072 | 1.7072 | 1.4938 | 2.5608 | 2.3474 |
| 28 | 1.7072 | 1.9206 | 1.7072 | 1.7072 | 1.7072 | 1.7072 | 1.9206 | 1.7072 | 2.5608 | 2.5608 |
| 30 | 1.7072 | 1.9206 | 1.7072 | 1.9206 | 1.7072 | 1.7072 | 1.9206 | 1.7072 | 2.3474 | 2.5608 |
| 34 | 1.7072 | 1.9206 | 1.9206 | 1.9206 | 1.7072 | 1.7072 | 1.9206 | 1.7072 | 2.3474 | 2.5608 |
| 48 | 1.7072 | 1.4938 | 1.9206 | 1.9206 | 1.7072 | 1.7072 | 1.9206 | 1.7072 | 2.3474 | 2.5608 |
| 52 | 1.7072 | 1.4938 | 1.9206 | 1.9206 | 1.7072 | 1.7072 | 1.9206 | 1.7072 | 2.3474 | 2.5608 |
| 58 | 1.7072 | 1.4938 | 1.9206 | 1.9206 | 1.7072 | 1.7072 | 1.9206 | 1.7072 | 2.3474 | 2.5608 |
| 72 | 1.7072 | 1.4938 | 1.9206 | 1.9206 | 1.7072 | 1.7072 | 1.9206 | 1.7072 | 2.3474 | 2.5608 |

*Cell ≤0.1g/l*
*Substrate=1g/l*
*Temp=37$^0$C*
*pH=4.5*

### Effects of Cell Loading

Glucose concentration in the degradation of orange mesocarp cellulose by T. reesei studied under different cell loading and at fixed substrate concentration. The result is presented in Table 2.The deductions from these results are: For each cell loading, there was an increase in glucose concentration. That is, there was a substantial increase in glucose concentration with increase in cell loading. Similar results on bioconversion of forest products for ethanol

production have been reported [3]. An exponential growth phase (the progressive doubling of cell number) resulting in a continually increasing rate of growth in the population [5] is seen from Table 2.

## CONCLUSION

As the substrate concentration increases from 0.2g/L to 2g/L, there was a corresponding increase in glucose concentration from 1.7mmol/L to 2.3mmol/L. It was also observed as well that, as the substrate concentration increases at a constant (fixed) cell concentration, there was a corresponding increase in glucose concentration.

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