

# UPSCALE OF THE EXTRACELLULAR GLUCOSE OXIDASE PRODUCTION IN *ASPERGILLUS NIGER* VAR *TUBINGENSIS* STRAIN ED8

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## Abstract

*Aspergillus niger* var *tubingensis* naturally produces the enzyme glucose oxidase. In previous studies it was discovered that the Ed8 strain is doing that remarkably well compared to other strains and microorganisms. As a continuation to these investigations, experiments with Ed8 had been done in a larger scale. The experiments show a high dependency of GOX production on aeration. Where in the small scale of 50 mL working volume (as used in previous studies) the specific oxygen transfer is sufficient, one has to use active aeration at higher working volumes. Also important is the agitation or more specifically the power entry per volume which affects the size of the Ed8 agglomerates. The agitation in the Airlift reactor seems not to be sufficient to effectively grow the Ed8 microorganism. To run the fermentation system on a large scale, one has to find the best parameter set of agitation and aeration to achieve as much and as small Ed8 agglomerates as possible.

## Resumen

*Aspergillus niger* var *tubingensis* produce naturalmente la enzima glucosa oxidasa. En estudios anteriores se descubrió que la cepa Ed8 ofrece alto desempeño en comparación con otras cepas y microorganismos. Como continuación a estas investigaciones, en este trabajo experimentos con Ed8 se hacen a una escala mayor. Los experimentos muestran una alta dependencia de la producción de GOX en la aireación. Mientras en la pequeña escala de 50 ml de volumen de trabajo (como se usa en estudios anteriores) la transferencia específica de oxígeno es suficiente, uno tiene que usar la aireación activa en mayores volúmenes de trabajo. También es importante la agitación o más específicamente la entrada de energía por volumen que afecta el tamaño de los aglomerados Ed8. La agitación en reactor AirLift no parece ser suficiente para crecer eficazmente el microorganismo Ed8. Para ejecutar el sistema de fermentación a gran escala, uno tiene que encontrar el mejor conjunto de parámetros de agitación y aireación para lograr tanto y tan pequeños aglomerados Ed8 como sea posible.

## Palabras Clave

GOX Production, Scaling-Up, Fermentation, Stirred Tank Reactors

## INTRODUCTION

This work follows a master thesis that was written to investigate glucose oxidase (GOX) production in *Aspergillus niger var tubingensis* strain Ed8 in a small scale (50 mL Flask) [1]. The superordinated topic is the reduction of chrome Cr(VI) to Cr(III). It was investigated in previous studies that GOX is playing a big role in reducing chrome in the Ed8 fermentation [2]. The strain Ed8 showed the highest rates of chrome reduction compared to other strains as well as greater secretion of GOX [1].

GOX is widely used in the industry as an additive to preserve food. The conversion of glucose can prevent caramelization and avoid deterioration by removing oxygen. It can also act as a preservative for milk because of the produced hydrogen peroxide which reduces microbial growth. It can be also used in the clinical area to determine glucose in biological fluids and to monitor glucose in fermentation processes. Due to its antimicrobial activity GOX is also used in tooth paste, lotions, shampoos, cosmetics, etc. The main product of GOX catalyzed reaction, gluconic acid, is used in chemical industries as a whitener and cleaner.

The task was to run the fermentation in a larger scale as well as trying piconcillo as a more economic substrate instead of purified glucose. The optimal parameters of the fermentation had to be found. Due to the time limitation and the fact that each fermentation runs 72 hours, there has to follow a deeper research on how the parameters affect the fermentation.

## MATERIALS AND METHODS

### List of devices

| Type                       | Name               | Manufacturer      |
|----------------------------|--------------------|-------------------|
| Flask (250 mL)             | -                  | Corning           |
| Stirred tank reactor (2 L) | Labfors 5          | Infors HT         |
| Airlift reactor (1.5 L)    | -                  | SEV               |
| Spectrophotometer          | Genesys 10S UV-VIS | Thermo Scientific |
| Microcentrifuge            | LSE 6765-HS        | Corning           |
| Precision scale            | PL403              | Mettler Toledo    |
| Micropipette 10-1000 µL    | Pipetman           | Gilson            |
| Microscope                 | Primo Star         | Zeiss             |
| Autoclave                  | CV300              | AESA              |
| Recirculator               | FC-10              | PRENDO            |

### Fermentation systems

- 250 mL flasks in a water bath shaker
  - Working volume: 50 mL
  - Agitation: translatory at 150 rpm
  - Aeration: none
  - 12 flasks are used to determine cell growth
- Stirred tank reactor (Labfors 5™)
  - Working volume: 2 L
  - Agitation: helical ribbon impeller at 150 or 250 rpm
  - Aeration: 5 L/min air
- Airlift reactor
  - Working volume: 1.5 L
  - Agitation: circulation through buoyancy of air bubbles
  - Aeration: 2-4 L/min air

### Medium

As a medium, a modified variant of Lee's minimal medium (LMM) [3] with varied glucose concentration, has been used.

1 L LMM contains:

|   |               |
|---|---------------|
| KH <sub>2</sub> PO <sub>4</sub>                 | 0.25 g        |
| MgSO <sub>4</sub>                               | 0.2 g         |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 0.5 g         |
| NaCl  | 0.5 g         |
| Glucose   | 2.5 g // 50 g |

(or replaced with piconcillo)

Citric acid 45 mM

The pH is adjusted to 5.3 with NaOH after complete dissolution. Then the medium gets sterilized in an autoclave at 121°C for 15-30 min.

### Storage and (sub-)cultivation of the Ed8 culture

The Ed8 spores are stored in distilled water at 4°C in a sterile 50 mL Falcon™ tube. To guarantee constant spore viability through all experiments, the Ed8 has to be subcultivated every month. The steps of subcultivation are as follows:

- Spread the spores out on PDA-Plates with a grease pencil
- Cultivate 4-6 days in an incubator at room temperature
- Resuspend the spores with distilled water
- Wash 2 times with distilled water by centrifugation (3000 rpm, 15 min)
- Determine cell concentration by cell counting in a Neubauer Improved™ counting chamber

### Start of the fermentation

To start the fermentation the inoculation is done with  $5 \cdot 10^5$  cells/mL. The fermentation is carried out at 28°C under most sterile conditions possible. The samples of 1 mL are taken in equidistant time intervals and stored in 1.5 mL microtubes.

### Biomass concentration measurement

To determine the dry biomass concentration in g/L, the fermentation broth first gets filtered. The filtrated biomass is stored in a drying oven until it reaches constant weight. The dry biomass gets weighted and the result is divided by the total working volume.

### Substrate concentration measurement

The glucose concentration measurement is done with the DNS-Test [4]: 3,5-Dinitrosalicylic acid gets reduced to 3-Amino-5-nitrosalicylic acid when reducing sugars are present (see Figure 1). DNS changes its color which is measurable at 540nm.

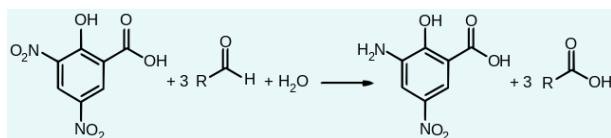


Figure 1: DNS reduction scheme. [Wikimedia Commons]

### Product activity measurement

Enzymatic activity measurement of GOX is done with an ABTS-peroxidase-linked assay (see Figure 2) [1]. GOX catalyzes the conversion of glucose to gluconic acid. When hydrogen peroxide is present, peroxidase will catalyze the ABTS oxidation. The color shift is measurable at 420nm.

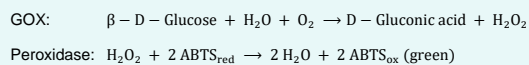


Figure 2: ABTS-peroxidase-linked assay

## RESULTS AND DISCUSSION

### Small scale fermentation

The first fermentation was carried out in 250 mL flasks with 50 mL working volume and 2.5 g/L glucose concentration (see Figure 3). The biomass concentration shows a lag-phase in the first 18 hours, then a log-phase until 60 hours and in the end a stationary phase. The end concentration of dry biomass was 2.74 g/L. There was no GOX activity detectable. The experiment was repeated with 50 g/L glucose (see Figure 4), which was defined as the optimum concentration for GOX production in previous studies [1]. The biomass concentration now was 17.33 g/L and 1.45 U of enzyme activity had been detected. The substrate is not used completely but the overshoot is needed to provide better growth rate and GOX production.

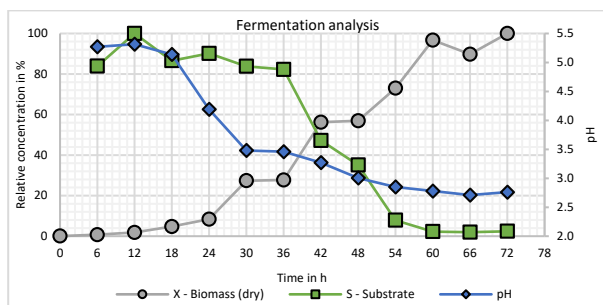


Figure 3: Fermentation analysis (2.5 g/L)

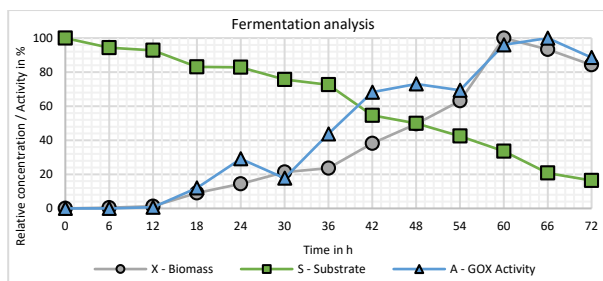


Figure 4: Fermentation analysis (50 g/L)

### Small upscale fermentation

The experiments had been repeated with 50, 100, 150 and 200 mL working volumes in 250 mL flasks (see Figure 5). With increasing working volume the substrate consumption and GOX production decrease drastically because the specific area of oxygen transfer gets less and less. The graph

shows the high dependency of the fermentation on sufficient aeration.

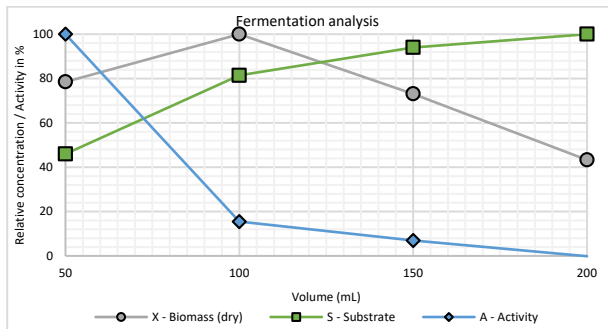


Figure 5: Fermentation analysis with increasing working volume

### Labfors 5<sup>TM</sup> fermentation

The Labfors 5<sup>TM</sup> is a stirred tank reactor with pH and temperature control. It was tried to provide the same conditions as in the small-scale fermentation. There had been done fermentations with and without aeration (see Figure 5 and 6). With aeration, two different revolutions per minute had been tested. Without aeration no enzyme activity and no substrate consumption could be detected. The end biomass concentration was very low at 3.20 g/L. After providing aeration, a curve similar to the small scale could be obtained at 250 rpm. The fermentation seems runs faster with 150 rpm but the result could not be verified yet. With both agitations, a maximum of ~2 U could be obtained. The production stops after 72 hours.

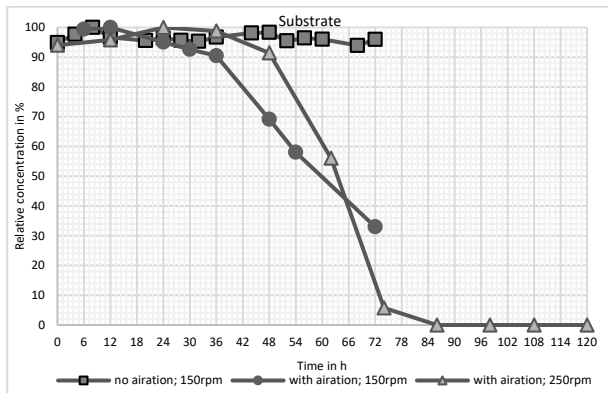


Figure 6: Labfors fermentation; Substrate concentration

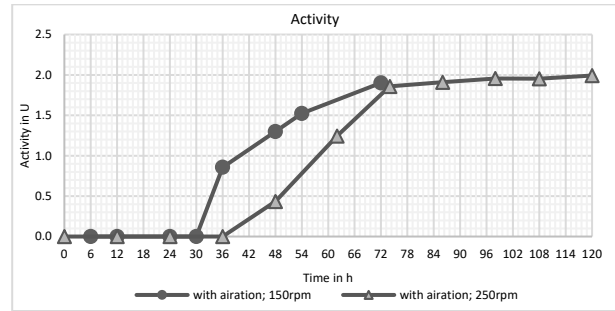


Figure 7: Labfors fermentation; Enzymatic activity

### Airlift fermentation

Since the motor of the Labfors 5<sup>TM</sup> got damaged, the system had to be transferred to the 1.5 L Airlift reactor, where fermentations with LMM and piloncillo had been analyzed (see Figure 5). The piloncillo seems to lead to higher enzyme production. However, these results are not representative because of difficulties with the circulation. The Ed8 globes got too big too quickly, so the system got stuck at about 48 hours. The results have to be therefore verified in future experiments.

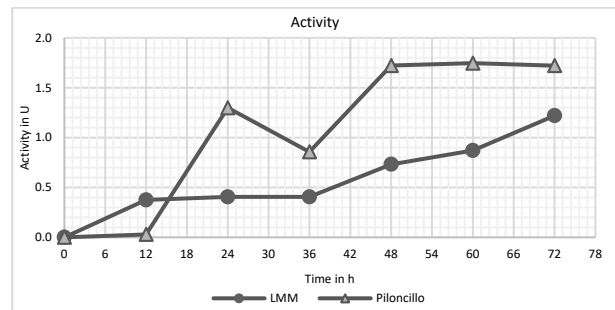


Figure 8: Airlift fermentation with LMM and piloncillo

### CONCLUSIONS

From the experiments one can make the following assumptions for an optimized upscale fermentation:

- The GOX production is highly depending on the right parameter set of agitation and aeration
- There has to be an overshoot of substrate to force higher growth rate and GOX production

In future investigations the perfect parameter set of aeration and agitation as well as the optimal substrate concentration have to be determined. It is

suggested to use a stirred tank reactor with a sufficient aeration system to provide optimal cell growth.

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