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Review

Production of cellulolytic enzymes from ascomycetes: Comparison of solid state and submerged fermentation

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ABSTRACT

Optimizing production of cellulose degrading enzymes is of great interest in order to increase the feasibility of constructing biorefinery facilities for a sustainable supply of energy and chemical products. The ascomycete phylum has a large potential for the production of cellulolytic enzymes. Although numerous enzymatic profiles have already been unraveled, the research has been covering only a limited number of species and genera, thus leaving many ascomycetes to be analyzed. Such analysis requires choosing appropriate media and cultivation methods that ensure enzyme profiles with high specificities and activities. However, the choice of media, cultivation methods and enzyme assays highly affect the enzyme activity profile observed. This review provides an overview of enzymatic profiles for several ascomycetes covering phylogenetically distinct genera and species. The profiles of cellulose degrading enzymes are correlated to the use of submerged culturing and solid state culturing. Even though submerged fermentation (SmF) is the most common method for commercial enzyme production, the use of solid state fermentation (SSF) is praised as a promising way of producing higher enzyme titers compared to SmF. Current comparisons of enzyme activities obtained from SmF and SSF do not account for all variables thereby complicating comparisons and diminishing credibility of conclusions being made. This review aims at providing guidelines for directly comparing cellulolytic enzyme production in SSF with SmF to advance future research of enzyme production.

Contents

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1. Introduction

One of the promising ways to achieve a sustainable and environmentally friendly energy source is the production of hydrocarbon fuels and bioethanol from lignocellulose [1,2]. There is, however, a lack of large scale industrial biofuel plants in Europe, probably due to challenges with financial viability, economies of scale and lack of political incentives. The economy is especially challenged by the expensive commercial enzymes needed to convert cellulose and hemicellulose in plant biomass into fermentable sugars [3]. In 2004 the market price of commercial cellulase from Alltech was set at $90/kg cellulase [4].

Conversion of plant biomass requires a mixture of different enzymes, including cellulases (e.g. endo-1,4-β-D-glucanase (EG), exo-β-1,4-glucan cellobiohydrolase (CBH) and β-1,4-glucosidase (BG)) as well as the hemicellulases (e.g. exo-1,4-β-xylosidase and endo-1,4-β-xylanase). These enzymes have primarily been derived from ascomycete fungi, in particular *Trichoderma reesei*, *Aspergillus niger* and *Talaromyces pinophilus* (*Penicillium pinophilum*) [5–7]. Commercial enzymes are mostly delivered as mixtures or blends composed of several enzymes, including all or most of the above mentioned enzymes. Such blends can be further optimized by tailoring them for specific biomasses. Tailored blends might also decrease costs pertaining to enzyme production by utilizing an optimal ratio between the enzymes and thereby lowering the enzyme usage [8]. By improving the method of enzyme production a higher yield may be achieved, which would also increase the feasibility of biorefineries. Novozymes, which is the world leading enzyme producer, currently applies submerged fermentation (SmF) for cellulase production. Also the American company Dyadic produces a liquid cellulase product through SmF of *Myceliophthora thermophila* as well as a cellulase powder from SmF of *Trichoderma longibrachiatum*.

Higher yields have been claimed when using solid state fermentation (SSF) [9]. It has also been claimed to be more economically advantageous e.g. for *Clostridium thermocellum* cellulase production in SmF and SSF where the unit costs based on simulated large scale production were calculated to be $40.36/kg cellulase and $15.67/kg cellulase, respectively [4]. The SSF process has been applied for industrial enzyme production of hemicellulases mainly in Japan, using the production organisms *Tr. viride*, *Tr. koningii*, *Tr. reesei* and *A. niger* [10]. Cellulase mixtures are also produced by SSF from the French company Lyven that uses the organisms *Tr. longibrachiatum*, *Rasamsonia emersonii* (previously *Talaromyces emersonii* [11]) and *A. niger*. Other ways to increase yield is to select a new fungal strain from a culture collection or nature with higher activity of the desired enzymes or to genetically engineer a current strain for increased production. Although the engineering of fungal strains is a common approach to increase enzyme production, it is still necessary to know whether the strain performs optimally in SmF or SSF. It is also possible to engineer the enzymes in order to increase their specific activity as well as temperature and pH stability [12]. Another approach is to reduce the amount of added enzymes as in consolidated bioprocessing (CBP) where the fungal strain simultaneously hydrolyze and ferment lignocellulosic biomass [13].

The purpose of this review is to provide an overview of the process from selection of fungal strains to selection of optimal production methods. The aim is to compare and evaluate the SmF and SSF processes for increased cellulolytic enzyme production, and to put forward recommendations for future enzyme production research. This review does not cover current methods for engineering of enzyme production strains nor will it provide description of alternative processes such as CBP.

2. Determination of cellulase activity

When it comes to quantification of cellulolytic activity, there are several different assays available. Comparisons between reports on enzyme activity are complicated by the use of different assays and units of activity. There are, however, standardized International Union of Pure and Applied Chemistry (IUPAC) approved methods for determining cellulase activity. These are the filter paper assay (FP), carboxymethyl cellulose (CMC) assay and cellobiase assay [14]. The FP assay has been further standardized by the national renewable energy laboratory [15]. Reproducibility of the CMC and FP assays is challenged by the use of 3,5-dinitrosalicylic acid (DNS) [16], which is used to determine the amount of reducing sugars (glucose) released from the cellulose substrates. This reagent requires boiling for full color development, which may result in partial degradation of reducing sugars. Also the FP assay is highly dependent on the amount of BGs in the enzyme blends being assayed [17]. It has been reported that adding supplemental BGs can increase the reproducibility of the FP assay [18]. With a determined amount of protein, activity is reported as U/g of protein, where U stands for enzymatic units corresponding to amount (μmol) substrate converted pr. minute. If the protein concentration is unknown, the activities will be reported as U/ml of added enzyme solution or as U/g carbon source or substrate (U/gs) in the medium. These three ways of reporting activity are not directly comparable. The activity is sometimes also reported as productivity in U per liter of enzyme solution per hour of production time (U/L/h), giving an idea of the overall potential for the process. In SSF the primary way of reporting activity is by U/gs; this can be used as an assessment of the required substrate to reach such enzyme activities with a given fungus. However, it cannot be used as a measure of productivity, which it is sometimes reported as, since it lacks the measure of time as well as amount of liquid. If the final product of cellulase from SSF is to be applied on-site without lixiviation or drying, as has been suggested [4], the productivity unit U/gs/h is relevant. The amount of added liquid for lixiviation is relevant only if the enzymes are to be freeze dried and used off-site.

The predominant hemicellulolytic enzymes are xylanases, the three best known of these are arabinofuranosidases, endo-xylanases and β-xylosidases. The available assays have variations in both assay conditions (e.g. temperature, duration of incubation or substrate employed) and in the principle of quantification of enzyme activity (e.g. reducing sugars released from substrate, amount of dye released from covalently dyed xylans and measurement of decreases in viscosity or turbidity) [10]. The most commonly used assays apply the measurement of released reducing sugars liberated from insoluble xylans in 1% solution. The reducing sugars can then be measured by either using DNS reagent [16] or the method of Somogyi-Nelson [19,20]. Furthermore, for increased sensitivity of sugar detection high performance liquid chromatography (HPLC) [21,22] or high performance anion exchange chromatography (HPAEC) can be used [23]. Measurement of monomer sugars via HPLC has been applied for analysis of carbohydrate content of liquid fractions obtained from biomass pretreatment [24]. By using insoluble xylans, these assays become unreliable due to the different degrees of polymerization and substitutions of the xylan polymers. Choice of substrate have been shown to account for variations between 20 laboratories amounting to a standard deviation of 108% from the mean [25].

Therefore, when attempting to compare enzyme activities across research papers hindrances appear with regards to the assay conditions used. Even when the standardized CMC or FP assays are applied, conditions such as temperature, pH and concentration of buffer as well as the amount of substrate vary between papers [26–30]. In order to obtain comparability between research papers,

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