



Liquefied wheat bran as carbon source and inducer in high-solids submerged cultivation of *Aspergillus niger* for xylanase production



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ABSTRACT

High-solids submerged cultivation for microbial production of industrially relevant enzymes can be facilitated by using a liquefied substrate as carbon source and inducer. Here, liquefied wheat bran (WB) was evaluated as carbon source and inducer in high-solids submerged cultivation (20%, w/w) of a filamentous fungus, *A. niger*, for xylanase production. A systematic study (one-factor-at-a-time) of the use of liquefied WB under different conditions of temperature, pH, enzyme loading, and reaction time was performed. This study revealed that xylanase production was favored by a substrate liquefied using the optimum pH (4.8) and temperature conditions (50 °C) of the commercial enzymatic preparation (2.8 FPU/g solids), while the liquefaction time was not significant above 6 h. The proposed strategy resulted in xylanase activity 5-fold higher than for a cultivation with non-liquefied substrate (control). These findings should contribute to the development of xylanase production processes using high solids loading submerged cultivation in conventional large-scale bioreactors.

1. Introduction

Xylanase enzymes have a wide range of applications in the food industry, as well as in other technological sectors such as pulp and paper production and feed manufacture (Collins et al., 2005). In the bioethanol sector, the action of xylanases improves cellulose conversion during the enzymatic hydrolysis of the biomass by increasing the accessibility of the substrate to cellulases (Gao et al., 2011; Kumar and Wyman, 2009). The endo-1,4- β -xylanase enzyme (xylanase) cleaves the 1,4- β -glycosidic linkage between xylose residues in the backbone of xylan and is essential for the depolymerization of hemicellulose (Dodd and K O Cann, 2009). On an industrial scale, filamentous fungi of the genera *Aspergillus* and *Trichoderma* are the main producers of xylanases (Park et al., 2002). Production of xylanases using strains of *Aspergillus* in both submerged fermentation (SmF) and solid-state fermentation (SSF) systems has been widely reported (Betini et al., 2009; Chapla et al., 2010; Chipeta et al., 2008; Fang et al., 2010; Ghanem et al., 2000; Pirota et al., 2013; Membrillo Venegas et al., 2013).

Despite the fact that most commercial xylanase enzymes (about 80–90%) are produced using SmF (Polizeli et al., 2005), the use of SSF is particularly advantageous for enzyme production by filamentous fungi. This is because it simulates the habitat of these microorganisms and,

from an environmental perspective, enables the use of agro-industrial residues as sources of carbon and energy for microorganism growth and enzyme production (Farinas, 2015). However, the implementation of SSF at an industrial scale has been held back, mainly due to difficulty in monitoring and controlling the different process variables. Therefore, the use of high loadings of lignocellulosic biomass for enzyme production in SmF systems seems to be an advantageous approach, in terms of the potential for large-scale implementation, due to well-developed SmF bioreactors, instrumentation, and process control.

In order to develop a bioprocess strategy to carry out SmF at high solids loading (>15%, w/v), certain limitations need to be addressed. The type and metabolism of the microorganism, as well as the characteristics of the substrate and the level of solids content, can lead to agitation and mass transfer problems during the cultivation, due to non-uniform mixing (Ioelovich and Morag, 2012; Skovgaard et al., 2014; Viamajala et al., 2009). Furthermore, the viscosity of the culture broth also increases during the cultivation for enzyme production by filamentous fungi, which can further impair uniform homogenization (Antecka et al., 2016).

One potential solution to the aforementioned problems is to perform liquefaction of the lignocellulosic material, prior to its use in the microbial cultivation system (Cunha et al., 2014). This strategy is

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especially promising for the production of enzymes secreted into the medium. In a study by Cunha et al. (2014), liquefied sugarcane bagasse was used as the cultivation medium for endoglucanase production by *A. niger*, resulting in a 12-fold increase in productivity, compared to SSF of non-liquefied bagasse. Enzymatic liquefaction leads to microfibril shortening and viscosity reduction (making it easy to agitate the lignocellulosic biomass suspension), with promotion of the release of sugar monomers and soluble oligomers (Thygesen et al., 2011) that are easily assimilated sources of carbon and inducers during microbial cultivation. Squinca et al. (2018) recently used enzymatically liquefied biomass as a culture medium for endoglucanase production by *A. niger* in SmF. The authors also applied the home-made enzymatic cocktail obtained from the SmF in the liquefaction step, thus closing the loop, in order to eliminate the need to purchase commercial enzymes. However, despite the promising results achieved using liquefied lignocellulosic material as inducer and carbon source during microbial cultivation, the development of high-solids loading cultivations for the production of different industrially relevant enzymes remains to be investigated. One interesting biomass for such bioconversion is wheat bran (WB) which is composed basically of (w/w): 12.1% water, 13.5–18.4% protein, 2.6–3.4% fat, 2.7–6.1% ash, and 50.4–79.1% carbohydrates (around 24.9% starch, 10.7–28.4% arabinoxylans, and 13.2% β -glucan) (Beaugrand et al., 2004; Dobrev et al., 2007; Dornez et al., 2006; Palmara-Adrados et al., 2005; Roberts et al., 1985). As WB contains high level of the xylan it is a potential substrate for xylanase production by microorganisms.

The purpose of this work was to evaluate the feasibility of using liquefied wheat bran as carbon source and inducer in high-solids submerged cultivation (20%, w/w) of a filamentous fungus, *A. niger*, for xylanase production. To this end, a systematic study was carried out to evaluate the effects on xylanase production of the conditions employed for liquefaction WB using a commercial enzymatic cocktail, considering variables including temperature, pH, enzymes loading, and reaction time.

2. Materials and methods

2.1. Microorganism

The *Aspergillus niger* A12 strain was obtained from Embrapa Food Technology (Rio de Janeiro, Brazil). The strain was kept at -18°C and was activated by incubation in slants of potato dextrose agar (PDA) medium for 4 day at 32°C . Suspensions of spores were prepared by the addition of 10 mL of Tween 80 (0.3%, v/v). Spore concentrations were determined using a Neubauer chamber.

2.2. Wheat bran

The wheat bran (WB, 150–1000 μm) was supplied by the Richard Saigh Indústria e Comércio (Brazil). It was stored at -5°C and dried at 35°C for 12 h prior to use.

2.3. Pre-culture medium and conditions

2.3.1. Culture medium

The culture medium used in the pre-culture and the SmF cultivation was prepared as described by Mandels and Sternberg (1976) and contained (w/v): 0.14% $(\text{NH}_4)\text{SO}_4$, 0.20% KH_2PO_4 , 0.03% CaCl_2 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.50% peptone, 0.20% yeast extract, 0.03% urea, 1.0% Tween 80, and 0.10% of a salt solution (5.0 mg/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6 mg/L of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.4 mg/L of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 2.0 mg/L of CoCl_2). In addition to the aforementioned components, glucose was included in the pre-culture and SmF cultivation media, at concentrations of 30 and 10 g/L, respectively.

2.3.2. Pre-culture preparation

The pre-culture for the SmF cultivation was initiated with a 10^7 spores/mL conidial suspension in 500 mL Erlenmeyer flasks containing 100 mL of nutrient medium. The incubation was carried out for 50 h, at 32°C and 200 rpm in a shaker. After this period, a volume of 5 mL (10%, v/v) of the pre-culture suspension was transferred to the cultivation medium for xylanase production.

2.4. Liquefaction of wheat bran

The WB liquefaction was carried out using a commercial enzymatic preparation containing cellulase and hemicellulase complexes (Cellic Ctec 3, Novozymes, Denmark). A mass of 10 g of WB (dry mass basis) was loaded into a 250 mL Erlenmeyer flask and was sterilized for 15 min at 121°C . After cooling, a 23.3 mL volume of 0.05 mol/L sodium citrate buffer (pH 4.8) containing the desired enzymes loading was added in order to achieve a solids concentration of 30% (w/w). The liquefaction reactions were carried out using different temperatures (30, 40, and 50°C) and times (0, 6, 18, and 24 h), under 200 rpm agitation. The liquefied material suspension was then sterilized for 15 min at 121°C , prior to use in the SmF cultivations. All the liquefaction experiments were performed in triplicate and the data were calculated as means \pm standard deviations.

2.5. Submerged fermentation

A volume of 5.0 mL of the pre-culture medium was inoculated into flasks containing the liquefied WB. In order to achieve a final solids loading of 20% (w/w), 11.7 mL of the nutrient medium was added to each flask. The cultivations were carried out for 72 h, at 32°C , in an orbital shaker incubator set at 250 rpm. After the cultivations, the suspensions were filtered and kept frozen until the analytical assays. All the cultivation experiments were performed in triplicate and the data were calculated as means \pm standard deviations.

2.6. Enzyme activity assay

The xylanase activity was measured according to the methodology described by Bailey and Poutanen (1989), using incubation of 1.0 mL volumes of the suitably diluted samples, with 1% beechwood xylan (Sigma, USA) solution as substrate, in 0.20 mol/L sodium acetate buffer at pH 5.0. The incubation was performed at 50°C for 30 min. One unit of xylanase activity corresponded to 1 μmol of xylose released per minute at pH 5.0 and 50°C . The xylanase activity was reported as international units (IU) per mass of dry WB. Measurement of cellulase activity in terms of filter-paper units (FPU) per milliliter of original enzyme solution was according to the methodology described by Ghose (1987). The assay was occurred at 50°C for 60 min with the incubation of the 500 μL enzymatic solution, 50 mg of filter paper n $^{\circ}$ 1 Whatman, and 1000 μL sodium citrate buffer 0.05 mol/L at pH 4.8. Quantification of the reducing groups was performed according to the dinitrosalicylic acid (DNS) method (Miller, 1959).

2.7. Total protein concentration

The total protein concentrations in the samples were determined by the Bradford (1976) method, using bovine serum albumin as a standard. Total protein was reported as the mass of protein (in mg) per gram of dry WB.

2.8. Statistical analysis

Tukey test was used for multiple comparisons between pairs at a 95% confidence interval ($p < 0.05$) of the enzymatic activity obtained in the fermentations with liquefied WB under different pH, enzymatic loading and time of liquefaction using the Minitab software version 16.2.1.

3. Results and discussion

3.1. Time profile of protein and xylanase production under SmF

A preliminary set of cultivations was carried out in order to investigate the time profile of xylanase production during cultivation of *A. niger* under SmF with liquefied WB (Fig. 1). For this set of cultivations, the WB was liquefied for 6 h at 30% (w/w), using an enzyme load of 2.8 FPU/g of solids.

The xylanase activity increased up to 72 h and then declined, while the total protein concentration was highest at 24 h and subsequently decreased. During the first 48 h of cultivation, there was probably a sufficient amount of carbohydrates that could be easily assimilated by the fungus, preventing it from secreting large amounts of xylanase enzymes. The increased enzymatic activity after 48 h of cultivation suggested that the easily assimilable nutrients were becoming scarcer, hence requiring the synthesis and secretion of enzymes in order to depolymerize the remaining biomass. A similar finding was reported by Robl et al. (2015), with xylanase production by *A. niger* DR02 being associated with exhaustion of the carbon sources in an aqueous solution of liquor from the hydrothermal pretreatment of sugarcane bagasse.

The cultivation time required for higher xylanase activity during SmF using the liquefied substrate (around 72 h) was similar to the cultivation time reported by Delabona et al. (2012) for *A. fumigatus* xylanases using wheat bran under SSF. Based on these results, a set of experiments was carried out using 72 h cultivation, in order to evaluate the effects on xylanase production of the temperature, pH, enzymes loading, and reaction time used in the WB liquefaction.

3.2. Influence of liquefaction temperature

The temperature is an important process variable that governs the degree of enzymatic hydrolysis during biomass liquefaction. Fig. 2 shows the production of total proteins and xylanases, after 72 h, for cultivations using WB that had been liquefied at different temperatures (30, 40, and 50 °C) as carbon source and inducer. The xylanase activity increased with increase of the liquefaction temperature, with cultivation using WB liquefied at 50 °C resulting in xylanase activity 2-fold higher than when WB liquefied at 30 °C was used. Furthermore, comparison with a control cultivation carried out using solid WB (without the liquefaction step) revealed that xylanase production using the liquefied material was up to 5-fold higher than for the control.

The higher xylanase activity obtained using WB liquefied at 50 °C was probably related to the higher concentrations of xylanase-inducing

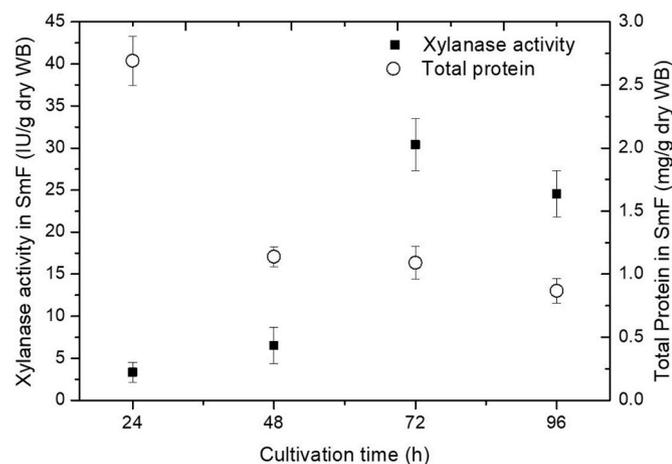


Fig. 1. Time profiles of xylanase production and total protein concentration during SmF of *A. niger* with liquefied WB. Liquefaction assay conditions: pH 4.8, 2.8 FPU/g, 6 h, 50 °C, and 200 rpm.

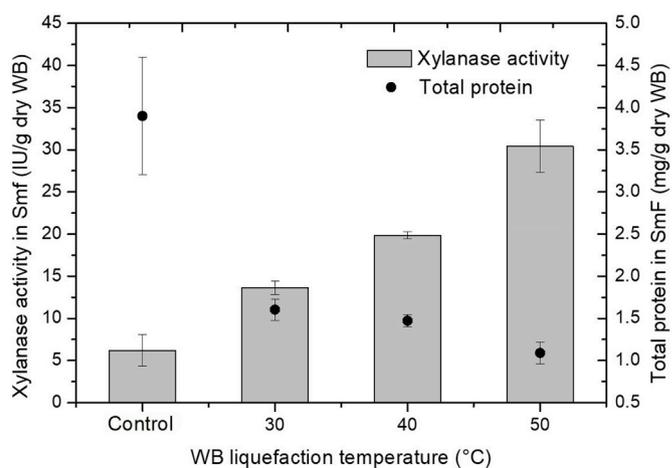


Fig. 2. Xylanase production in SmF of *A. niger* for 72 h, using WB liquefied at different temperatures. Control: cultivation carried out using non-liquefied WB. Liquefaction assay conditions: pH 4.8, 2.8 FPU/g, 6 h, and 200 rpm.

soluble sugars (glucose and xylose) and soluble oligosaccharides resulting from the more efficient biomass hydrolysis at this temperature. A temperature of 50 °C is within the range for optimum activity of the commercial enzymatic cocktail (Novozymes, 2012).

On the other hand, a higher concentration of total proteins was obtained in the control assay (Fig. 2), compared to the cultivations with liquefied WB, which was probably related to the complexity and composition of the non-liquefied solid substrate. Moreira et al. (2012) reported that the diversity of enzymes synthesized by the microorganism is proportionally induced by the complexity of the lignocellulosic substrate.

3.3. Influence of liquefaction pH

The influence on xylanase production of the pH employed for the WB liquefaction was evaluated by carrying out cultivations using the liquefied medium without further pH adjustment and with adjustment to pH 5.0 prior to the cultivations (Fig. 3). Higher xylanase production was achieved when the initial pH of the cultivation medium was between 4.8 and 5.4. Such pH conditions are within the optimal pH range of 4.75–5.25 for the commercial enzymatic cocktail used for WB liquefaction (Novozymes, 2012). Therefore, these results supported the previous findings for the effect of temperature, indicating that the conditions that favored WB hydrolysis resulted in the release of a greater quantity of xylanase inducers.

The total proteins concentration (Fig. 3b) was insensitive to liquefaction pH when the cultivation medium pH was set at 5.0. However, for the cultivations carried out with no pH adjustment of the liquefied WB, higher protein production occurred when liquefaction was carried at pH 5.2, similar to the condition observed for higher xylanase activity.

3.4. Influence of liquefaction time and enzyme loading

Investigation was also made of the influence of the WB liquefaction time and the enzyme loading used for production of xylanase (Fig. 4). The use of a higher enzyme loading for WB liquefaction (8.2 FPU/g) resulted in a 3-fold increase in xylanase production, compared to the control using liquefied WB (0 h). However, the liquefaction time, longer than 6 h, showed no influence on the production of xylanases. This indicated that a short liquefaction time (6 h), with a higher or even intermediate enzyme loading (2.8–8.2 FPU/g), could produce liquefied WB that was more suitable for inducing the production of xylanases.

The presence of an easily accessible carbon source at the beginning of the cultivation can contribute to a higher fungal cell mass, but when the

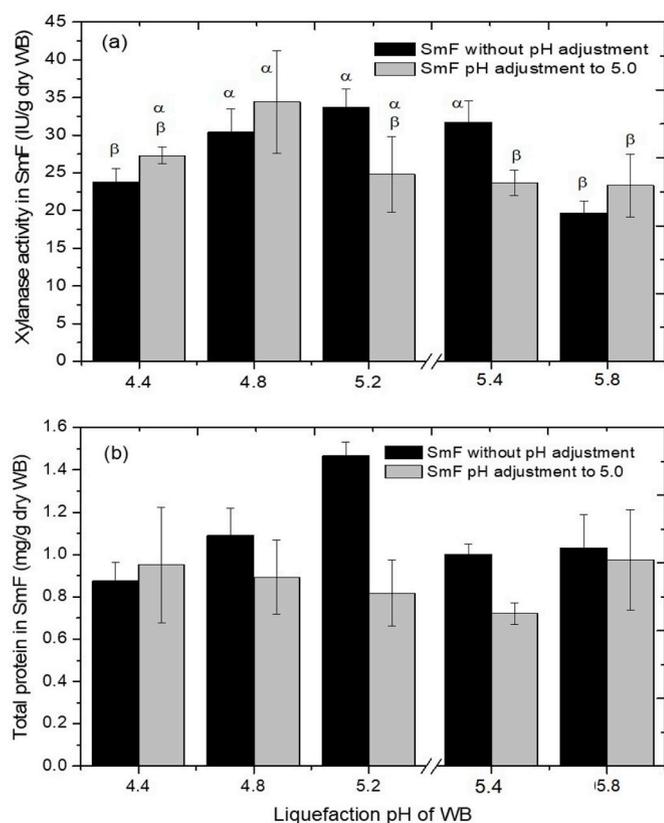


Fig. 3. Xylanase production (a) and total protein concentration (b) after 72 h of SmF of *A. niger* using WB liquefied at different pH values. Liquefaction assay conditions: 2.8 FPU/g, 6 h, 50 °C, and 200 rpm. Equal greek letters represent statistically similar values, according to the Tukey test, $p < 0.05$.

cultivation occurs at high glucose concentration catabolite repression make take place reducing enzyme production (Kobakhidze et al., 2016). Therefore, the reducing sugar concentration in the liquefied media used as carbon source and inducer in these cultivations were quantified (Table 1). The reducing sugars release profile varied similarly to the xylanase activity (Table 1, Fig. 4). At the higher enzyme loading (8.2 FPU/g), there was no increase in the concentration of reducing sugars (72.9 g/L) when liquefaction time increased from 18 to 24 h (Table 1). The stagnation in the reducing sugar release during a long liquefaction time of the may be due to the enzyme inhibition and deactivation, for instance by presence of phenolic compounds (Ximenes et al., 2011). Therefore, enzymes loading and liquefaction time must be carefully determined in order to avoid enzyme inhibition and to have some residual polymerized material to initiate and maintain the enzyme production by fungi.

Physical changes of the WB liquefied using an intermediate enzyme loading (2.8 FPU/g) and different hydrolysis reaction times were registered (Fig. 5). There was transformation of the wet solid particulate system into a dense suspension of fine particles. Considering that a higher solids loading increases the need for efficient homogenization during microbial cultivation (Ward et al., 2005), the results showed that the use of a high-solids loading of liquefied material (20%, w/w) should facilitated homogenization of the medium and provided a source of carbon and inducer that resulted in improved xylanase production. Such favorable results could be also attributed to the fact that the liquefaction promoted a better interaction between the fungal cells and the substrate. This was in agreement with the findings of Michelin et al. (2012), who reported that xylanase production by *A. ochraceus* using a combination of WB and hemicellulosic liquor derived from the auto-hydrolysis of wheat straw was 1.7 and 3 times higher than the production obtained using only WB or liquor, respectively.

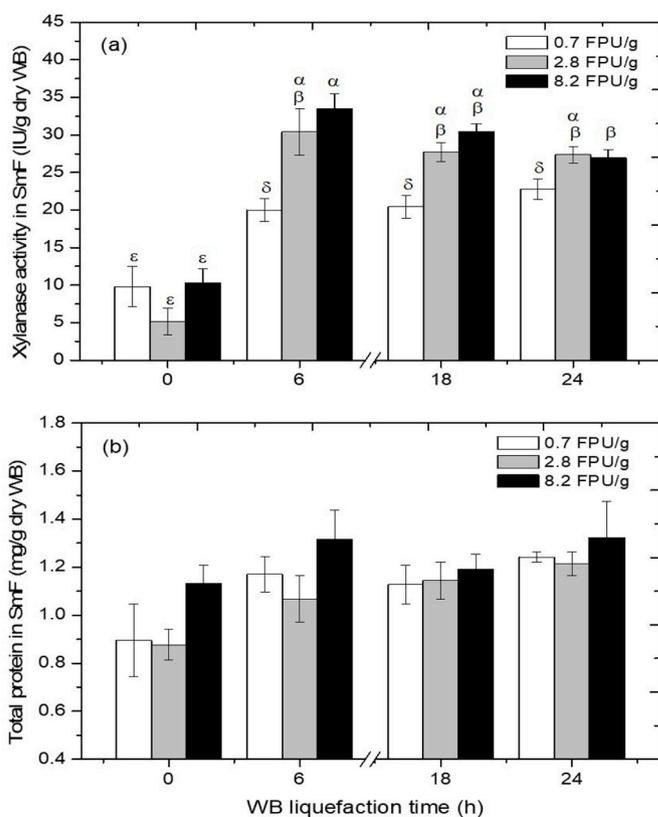


Fig. 4. Xylanase production (a) and total protein concentration (b) after 72 h of SmF of *A. niger* using wheat bran WB liquefied using different hydrolysis reaction times and enzyme loadings. Liquefaction assay conditions: pH 4.8, 50 °C, and 200 rpm. Equal greek letters represent statistically similar values, according to the Tukey test, $p < 0.05$.

Table 1

Concentrations of released reducing sugars in the supernatants of the liquefied WB produced using different liquefaction times and enzyme loadings. Liquefaction assay conditions: pH 4.8, 50 °C, and 200 rpm.

Liquefaction time (h)	Commercial enzyme loading (FPU/g of solids)	Reducing sugars concentration (g/L)
6	0.7	19.4 ± 0.8
	2.8	42.2 ± 6.3
	8.2	40.2 ± 3.1
18	- ^a	11.4 ± 0.4
	0.7	42.2 ± 3.4
	2.8	55.7 ± 2.1
	8.2	72.9 ± 0.8
24	- ^a	20.0 ± 2.1
	0.7	40.3 ± 3.6
	2.8	64.3 ± 2.4
	8.2	72.9 ± 3.4
	- ^a	19.6 ± 1.6

^a Without addition of enzymes (buffer only).

4. Conclusions

This study demonstrated the feasibility of using liquefied wheat bran at high-solids loadings (20%, w/w) as a strategy for improving efficiency of xylanase production by *A. niger* under batch submerged cultivation. Under the best liquefaction condition – 2.8 FPU/g of solids with the releasing of 42.2 ± 6.3 g/L of reducing sugars at 50 °C – the xylanase activity obtained (30.4 ± 3.1 IU/g dry WB) was up to 5-fold higher than for control cultivation with non-liquefied WB. Xylanase production was favored by use of a substrate liquefied using the optimum pH and temperature conditions of the commercial cellulolytic cocktail, while the

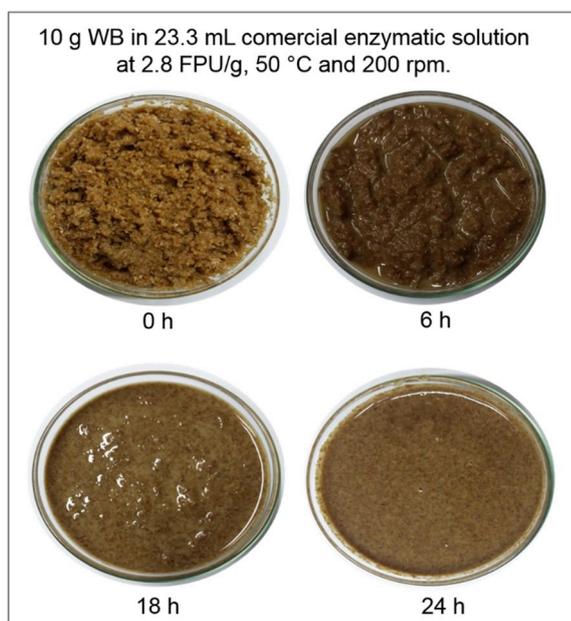


Fig. 5. Illustration of the physical changes of WB liquefied using an enzyme loading of 2.8 FPU/g, at different times of the hydrolysis reaction. Liquefaction assay conditions: pH 4.8, 50 °C, and 200 rpm.

liquefaction time was not significant above 6 h. This approach should contribute to implementation of cultivations at high solids loading in the existing large-scale submerged bioreactors, since it provides a specific inducer and favors fluidity of the cultivation medium.

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