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Short Communication

Polyphenols content of spent coffee grounds subjected to physico-chemical pretreatments influences lignocellulolytic enzymes production by *Bacillus* sp. R2

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HIGHLIGHTS

- Spent coffee grounds were used to produce lignocellulolytic enzymes by *Bacillus* sp. R2.
- Untreated sent coffee grounds were the best inducers for cellulase and pectinase.
- Boiling water/EDTA treatment induced highest xylanase and peroxidase activities.
- Untreated spent coffee grounds presented the highest content of polyphenols.
- Cellulase and pectinase correlated strongly with polyphenols content.

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ABSTRACT

The objective of this study was to investigate the impact of polyphenols content changes issued after physico-chemical treatments of spent coffee grounds on lignocellulolytic enzymes production by *Bacillus* sp. R2. Total polyphenols of the collected substrates were extracted with water under autoclaving conditions. Results showed that polyphenols content of spent coffee grounds decreased with continued treatments. Untreated spent coffee grounds were the best substrate for cellulase and pectinase (1.33 ± 0.06 μ/ml and 0.32 ± 0.02 μ/ml respectively). A strong positive correlation was noticed between polyphenols content and cellulase and pectinase activities. However, xylanase and peroxidase correlated moderately with polyphenols content and their highest activities were registered with spent coffee grounds treated with boiling water and 1% EDTA (0.31 ± 0.002 μ/ml and 15.56 ± 0.56 μ/ml respectively). The obtained results indicate that polyphenols content of the pretreated substrates influences the production of lignocellulolytic enzymes by *Bacillus* sp. R2.

1. Introduction

Spent coffee grounds (SCG) are a waste produced from brewing coffee. The industry of instant coffee produces 6 million tons of wastes each year (Mussatto et al., 2011), a rate which tends to rise in order to meet the market demand. Although several studies have been devoted to valorise this waste as a source for biofuels, phenolic compounds production, bioremediation and composting (Mussatto et al., 2011); SCG has not been the subject of study for the production of lignocellulolytic enzymes. The richness of SCG with organic compounds especially cellulose, hemicellulose and lignin, but also secondary metabolites such as polyphenols, and alkaloids (Pujol et al., 2013); enable it to be a suitable source for lignocellulolytic enzymes and other valuable compounds production.

Every year, billions of tons of lignocellulosic materials such as sugarcane bagasse, wheat bran and other residues are produced. This low-cost biomass is already used in the production of lignocellulolytic enzymes especially cellulase (Grigorevski-Lima et al., 2013). However, the complexity of lignocellulose reduces microorganisms' performance which makes resort to pretreatment techniques necessary to overcome this problem (Ravindran and Jaiswal, 2016). Pretreatment techniques can be physical, chemical, physico-chemical, biological and can also be combined (Ravindran

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and Jaiswal, 2016). This treatments aims to disrupt the compact structure of plant cell wall to enhance the enzymatic activities (Jönsson and Martín (2016)).

Despite the advantages of pretreatments; there is a side effect which is the liberation of lignocellulose-derived by-products that have inhibitory action on enzymes production and activities. Among the by-products released, there are phenolic and aliphatic compounds, furans, acetic acid, aldonic and aldaric acids (Jönsson and Martín (2016)).

The objective of this study was to investigate the effect of the polyphenols content changes of spent coffee grounds caused by several physico-chemical treatments on lignocellulolytic enzymes production by *Bacillus* sp. *R2*.

2. Material and methods

2.1. Microorganism and culture conditions

Bacillus sp. *R2*, isolated from Red sea and registered in GenBank database, with the accession number DQ923161, was used in the present study. This strain was selected for the present study based on its capacity to grow on lignocellulosic wastes and its multiple enzymes production especially cellulase and peroxidase (Khelil et al., 2015). Microorganism activation was carried out on nutrient broth media for 18 h at 30 °C under continuous shaking at 150 rpm.

2.2. Substrates preparation

Spent coffee grounds (SCG) were collected from a local cafeteria (Oran, Algeria); they were oven dried at 70 °C until constant weight. Dried SCG were then sieved to obtain a powder of (250–500 μm) and conserved at room temperature protected from moisture by a plastic wrap until use.

Preparation of substrates from SCG was done according to Chaa et al. (2008), it involves five steps. The first step consists to treat 20 g of SCG in a mixture of chloroform and methanol (150/150 v/v) for 14 h, then in pure ethanol for 2 h and finally in boiling ethanol for 2 h. In the second step, the obtained insoluble residue was treated in boiling distilled water for 4 h, and finally in 1% EDTA pH 6.8 at 80 °C for 4 h. The next step, the insoluble residue was treated in 1% NaOH prepared in 70% ethanol at 80 °C for 2 h. Finally the last step consisted to treat the insoluble residue with 14% KOH for 14 h at room temperature. After each step, the insoluble residue was washed several times with distilled water, dried at 70 °C and a sample (1 g) was conserved for further analysis. The collected substrates received codes from S1 to S5.

2.3. Determination of polyphenols content

Substrates collected from pretreated SCG were subjected to autoclave-extraction to simulate the fermentation medium. 0.1 g of each substrate was suspended in 10 ml distilled water and autoclaved for 20 min at 121 °C corresponding to 1 bar, after that, the suspensions were filtered and the filtrates were used for polyphenols content determination. Total polyphenols were determined by Folin-Ciocalteu method (Aguilar-Garcia et al., 2007). 50 μl of substrate extract was mixed with 1.25 ml of 10 fold diluted Folin-Ciocalteu phenol reagent. After 2 min of incubation at room temperature, 1 ml of 7.5% sodium carbonate was added and the mixture was incubated for 15 min at 50 °C. A calibration curve of gallic acid was established and the measurement was done at 760 nm. Total polyphenols values were expressed as milligram gallic acid equivalent per dry weight material (mg GAE/g SCG).

2.4. Enzymes production

Enzymes production was carried out in erlenmeyer flasks containing 1% of the collected substrates separately, peptone 0.1%, and beef extract 0.1%, pH 7. The flasks were inoculated with a 5% (v/v) of *Bacillus* sp. *R2*. The cultures were maintained at 37 °C under continuous shaking at 150 rpm. At an interval of 24, 48 and 72 h of incubation, culture broths were centrifuged at 10,000 rpm for 10 min at 4 °C and supernatants were conserved at −20 °C for further tests.

2.5. Enzyme assays

Total cellulase activity (FPA) was determined by the filter paper assay as described by Ghose (1987) using Whatman No. 1 filter paper as substrate. Xylanase activity was assayed according to Bailey et al. (1992). 0.5 ml of 1% oat spelt xylan (sigma) was added with 0.5 ml of supernatant. The mixture was incubated at 37 °C for 30 min. Pectinase activity was assayed by the dinitrosalicylic acid (DNS) method (Miller, 1959) using pectin from apple (sigma). 0.5 ml of 1% pectin was added with 0.5 ml of supernatant. The mixture was incubated at 37 °C for 60 min. To eliminate any interference between the color of reactions and the color of crude enzymes; delta absorbance was calculated by subtraction of the absorbance sum of enzymes controls and substrates controls. One unit of Fpase, xylanase and pectinase activities were defined as 1 μmol of reducing sugar released per minute under assay conditions.

Peroxidase activity was assayed using 2,2′-azino-bis (3-ethyl benzthiazoline-6-sulfonic acid) (ABTS) as a Substrate according to Sigma Aldrich (1996). Reaction mixture contained 2.9 ml ABTS reagent (9.1 mM ABTS in 100 mM Potassium Phosphate Buffer, pH 7.0 at 37 °C) and, 0.05 ml supernatant, while the blank contained 2.9 ml ABTS reagent and 0.05 ml Enzyme diluent (40 mM Potassium Phosphate Buffer with 0.25% (w/v) Bovine Serum Albumin and 0.5% (w/v) Triton X-100, pH 6.8 at 37 °C). The reaction was launched by mixing 0.1 ml of H_2O_2 0.3% (v/v) with the reaction mixture and the blank and recording the increase of the absorbance at 405 nm for 2 min. One unit of peroxidase activity was defined as 1 μmol of 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) released per minute under assay conditions.

2.6. Statistical analysis

All experiments were conducted in triplicate and the data was presented as the mean value ± standard deviation (SD). The relationship between enzymes production and polyphenols contents was investigated by calculating the correlation coefficient *R*. *P*-values of less than 0.1 were considered to be statistically significant.

3. Results and discussion

3.1. Effect of physico-chemical pretreatments on SCG polyphenols content

Polyphenols content of SCG substrates are presented in Table 1. It appears that the use of a mixture of chloroform and methanol coupled to ethanol treatments caused a loss of 52% in polyphenols content compared to untreated SCG. Pretreatment with organic solvent aims to eliminate liposoluble compounds such as waxes and pigments (Chaa et al., 2008) but also polyphenols and other compounds. Akowuah et al. (2005) found that chloroform gave the highest amount of polyphenols. In fact, polar solvent such as

Table 1
Effect of physico-chemical treatments of spent coffee grounds on polyphenols content.

Substrate	Treatment	Polyphenols content (mg GAE/g SCG)
S1	Untreated SCG	32.38 ± 0.13
S2	S1 + Chloroform–Methanol (v/v)/Ethanol	15.60 ± 0.09
S3	S2 + Boiling water/EDTA 1% pH 6.8 at 80 °C	13.52 ± 0.36
S4	S3 + 1% NaOH in 70% ethanol	09.73 ± 0.23
S5	S4 + KOH 14%	08.10 ± 0.10

acetate/methanol/water are efficient for polyphenols extraction (Hayouni et al., 2007).

It has to be noted that even after treatments with EDTA; NaOH and KOH which aim to remove pectins, lignins and hemicelluloses respectively; polyphenols were not totally eliminated despite the regression registered (75% of loss in the end of treatments). Previous studies on SCG; showed that the rate of polyphenols is between 16 mg GAE/g SCG and 25 mg GAE/g SCG (Mussatto et al., 2011; Zuorro, 2015). The value obtained in the present study showed a higher rate (32 mg GAE/g SCG) which is probably due to the autoclave-extraction.

3.2. Enzymes activities

Activities of cellulase, xylanase, pectinase and peroxidase were recorded after 24 h, 48 h and 72 h of *Bacillus* sp. *R2* incubation with SCG substrates collected from different stages of treatments. For all enzymes activities, the highest level was obtained after 72 h of incubation (Fig.1).

The highest activity of cellulase was registered with untreated spent coffee grounds (Fig. 1a). After chloroform/methanol coupled to ethanol treatment which aims to remove liposoluble compounds; the production of cellulase decreased by 25% and continued to decline with pursuing of treatment to reach a loss of 41%. The same trend was noticed with pectinase production (Fig. 1c) with similar loss rates between the beginning (22%) and the end of treatment (42%). In a similar study, Grigorevski-Lima et al. (2013) reported that untreated sugarcane bagasse was the best inducer for cellulase and xylanase produced by *Trichoderma atroviride* 676 when compared to sugarcane bagasse treated with steam explosion.

However, for xylanase and peroxidase productions (Fig. 1b,d); untreated SCG also induced an important production of enzymes but the highest level was registered with the substrate collected in the third step of treatment. In fact, treatment with hot water and EDTA affects the cell wall structure by detaching pectins which are either high-methylated so soluble in hot water; or low-methylated which are cross-linked by calcium bridges; requiring EDTA treatments that chelates calcium ions. By removing pectins, lignins and hemicelluloses became more accessible for the bacteria, inducing higher activities of peroxidase and xylanase. Pakarinen et al. (2012) found that removal of pectins enhance the enzymatic accessibility by increasing the availability of the cell wall substrate surface area.

3.3. Correlation between enzymes production and polyphenols content

The effect of polyphenols content on cellulase, xylanase, pectinase and peroxidase was assessed by calculating the correlation coefficient. Results are shown in Fig. 2. With regard to cellulase and pectinase; the values obtained were: 0.99 and 0.98 respectively (p-values of less than 0.01) which represent a strong positive linear relationship. However, xylanase and peroxidase correlated moderately with polyphenols content ($R = 0.56$ and 0.63 respectively, p-value of less than 0.1). The obtained results were contrary to the expectations. Many studies reported that phenolic compounds have inhibitory effect on lignocellulolytic enzymes by deactivating enzymes (Sineiro et al., 1997; Ximenes et al., 2011). While, in this study decrease in cellulase, and pectinase was closely

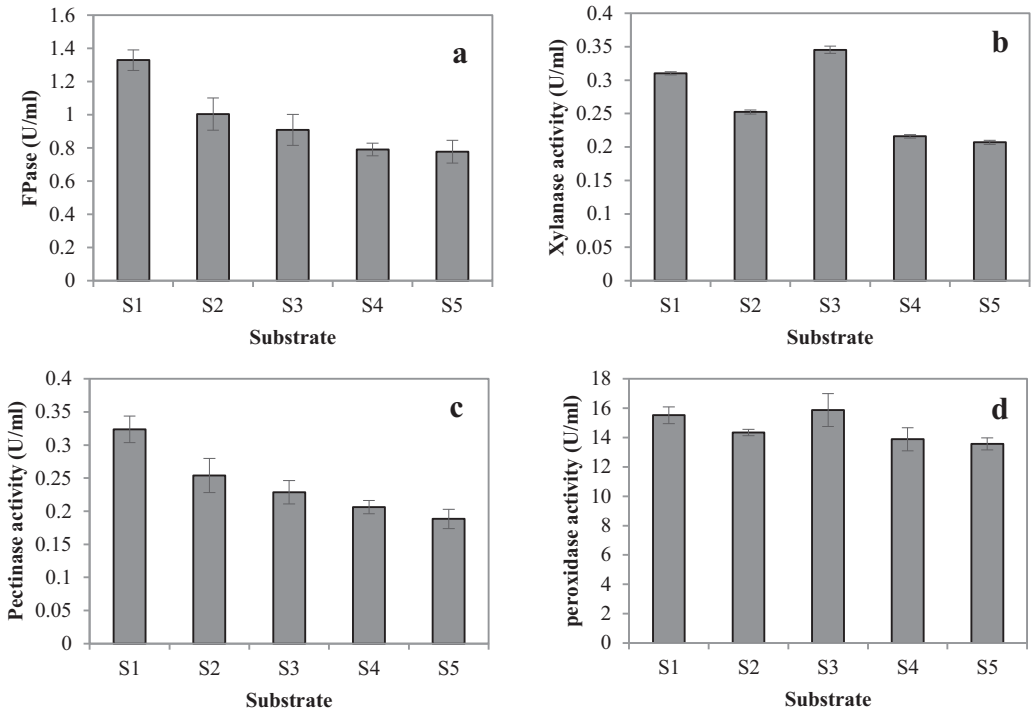

Fig. 1. Enzymes activities from *Bacillus* sp. R2 during 72 h of fermentation using substrates collected from spent coffee grounds subjected to physico-chemical treatments: (a) FPase activity, (b) Xylanase activity, (c) Pectinase activity and (d) Peroxidase activity.

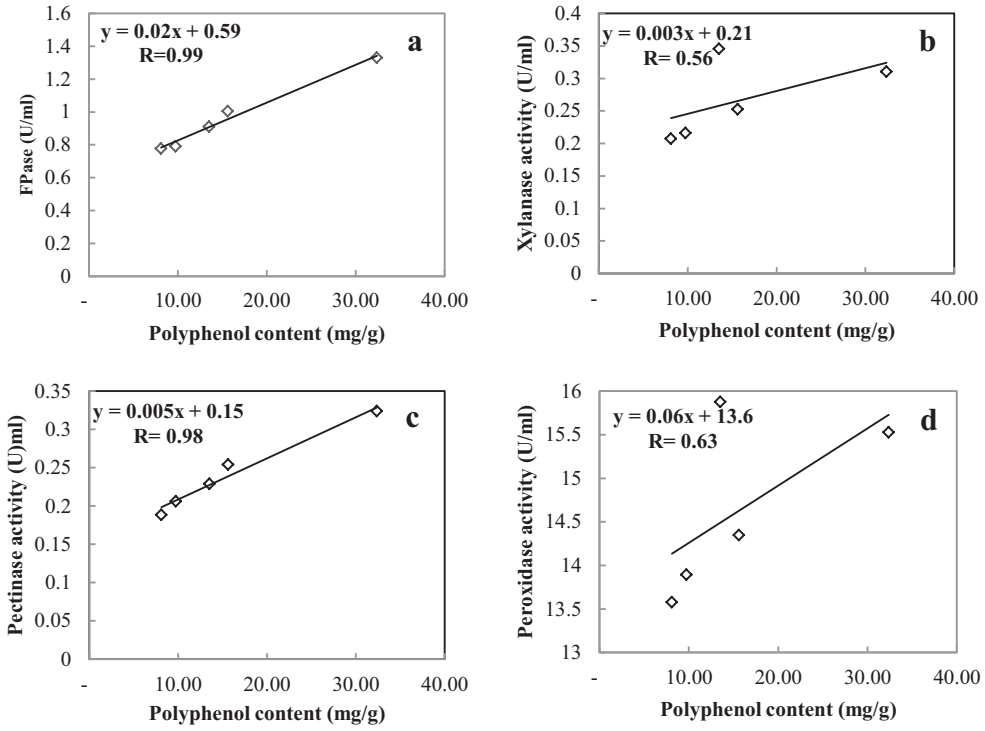

Fig. 2. Correlations of polyphenols contents of substrates collected from spent coffee grounds subjected to physico-chemical treatments to enzymes activities: (a) FPase (p-value is <0.01), (b) Xylanase (p-value is <0.1), (c) Pectinase (p-value is <0.01) and (d) Peroxidase (p-value is <0.1).

associated with the decrease in polyphenols amount. Olsen et al. (2011) suggested that the addition of non-ionic surfactants disrupt the enzyme-polyphenols interactions by a mechanism of strong polyphenols-non-ionic surfactants interaction. This leads to suggest that *Bacillus* sp. *R2* has produced biosurfactant which resulted in canceling the inhibitory effect on the enzymes production. Many studies reported the production of biosurfactant from *Bacillus* strains (Mnif and Dhouha, 2015). Some microorganisms are able to accumulate lipids like vegetable oils from the medium and produce lipopeptide surfactants (Mnif and Dhouha, 2015). Treatment with organic solvent that underwent SCG in this study has eliminated liposoluble compounds which are necessary for the production of biosurfactant; this may explain the decrease of the enzymatic production after the first treatment. Further investigations concerning the role of lipopeptide surfactants produced by *Bacillus* strains in the elimination of the inhibitory effect of polyphenols are needed.

4. Conclusion

The effect of polyphenols content of substrates collected from spent coffee grounds pretreatments on cellulase; xylanase; pectinase and peroxidase production from *Bacillus* sp. R2 was investigated. The results clearly indicated that untreated spent coffee grounds were a good substrate for the production of lignocellulolytic enzymes. Polyphenols content of the substrates correlated strongly with cellulase and pectinase and moderately with xylanase and peroxidase activities.

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