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Feruloyl Esterase Activity from Coffee Pulp in Solid-State Fermentation

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Summary

Hydroxycinnamic acids (HAs) have a potential application in the food and pharmaceutical industry because they are rich in phenolics. Feruloyl esterases release phenolic compounds from plant cell walls. Coffee pulp is rich in HAs linked to polysaccharides. A solvent extraction of free HAs was performed with aqueous methanol (80 %). A response surface methodology was applied to optimise the extraction of these compounds from coffee pulp, and the best results were obtained at 56 °C for 34 min. Alkaline and acid hydrolyses were performed to evaluate the content of linked HAs. Treated (extracted) coffee pulp was used to produce feruloyl esterases in solid-state fermentation by *Aspergillus tamarii* V12307, previously selected by a hydrolysis plate assay. Different dilutions of a culture medium were added to the coffee pulp, and the diluted medium with half the nutrients allowed for higher CO_2 production. A specific growth rate (μ_{CO_2}) of 0.25 h^{-1} and a lag phase (t_{lag}) of 14.3 h were observed under the selected conditions. Finally, enzymatic activities were 14.0 and 10.8 nkat per g of dried matter when methyl and ethyl ferulate were used as substrates, respectively. Productivities (9.3 and 7.2 nkat per g of dried matter per day, respectively) were higher when compared to other studies carried out in solid-state fermentation. Utilisation of coffee pulp for enzyme production improves the added value of this abundant by-product of the coffee industry.

Key words: coffee pulp, feruloyl esterase, hydroxycinnamic acids, solid-state fermentation, solvent extractions

Introduction

Food industry processing generates millions of tonnes of by-products per year. In coffee-producing countries, the production of coffee pulp (CP) is very important. CP is a residue rich in carbohydrates (on dry mass base around 40–50 %) and has a protein content of around 10 % (*1,2*). A promising group of natural compounds present in the cell wall of CP are hydroxycinnamic acids (HAs). These compounds are particularly valuable because of their antioxidant activity, and can be used in the pharmaceutical, cosmetic and food industries. This group of phenolic compounds is found in almost every plant. The

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Abbreviations: FA: ferulic acid, CA: caffeic acid, *p*CA: *p*-coumaric acid, ChA: chlorogenic acid, CP: coffee pulp, SSF: solid-state fermentation, FAE: feruloyl esterase, HAs: hydroxycinnamic acids

major representative of HAs is caffeic acid (CA), which occurs in foods mainly as an ester with quinic acid called chlorogenic acid (ChA, 5-caffeoylquinic acid). Other compounds like *p*-coumaric (*p*CA) and ferulic acids (FA) are present in dietary sources (*3*); these compounds are bonded through an ester linkage to the arabinoxylans of Gramineae (monocotyledons) or to the pectins of dicotyledons (*4*,*5*). On the other hand, 3,4-dimethoxycinnamic and sinapic acid are encountered less frequently (*6*). Particularly, FA and *p*CA are found covalently linked to polysaccharides by ester bonds and to lignin components by ester or ether bonds; this characteristic limits the extraction processes, and alkaline or acid hydrolysis are applied to release these compounds (*7*).

Enzymatic release of HAs is possible with feruloyl esterases (EC 3.1.1.73, carbohydrate esterase family 1), members of the carboxylic ester hydrolases which cleave the ester linkage between HAs and sugars (*5*,*8*,*9*). Cell wall complexity requires the synergistic interaction of a feruloyl esterase and hydrolytic enzymes acting on the cell wall polysaccharides (xylan or pectin). FA and *p*CA are bound through an ester linkage to the dicotyledon pectins (*4*) such as in coffee plants. CP contains around 6.5 % pectin in dry mass (*10*); in this case, a pectinase is necessary for cell wall degradation. Enzymatic or solvent extraction of HAs from natural sources depends on their composition, as well as the structure and physicochemical properties (*11*), so the extraction conditions should be designed and optimised (*12*).

Response surface methodology (RSM) coupled with the central composite design (CCD) is one of the most used optimisation procedures. This tool can help determine the optimal conditions that improve the extraction of free HAs from CP. Inhibition of the enzymatic activity of feruloyl esterases in the presence of CA and *p*CA has been reported (*4*), so a selective extraction of free HAs is required for biotechnological applications of CP, in particular for enzyme production. Solid-state fermentation (SSF) is applied for the treatment of agro-industrial residues to recover added-value products and enzyme production (*13*). The use of agro-industrial by-products as a carbon and energy source offers the advantage of combining the use of a cheap substrate and an interesting way of improving their added value. The aim of this work is the optimisation of a selective extraction of free HAs from CP, using the SSF with a fungal strain to produce feruloyl esterase.

Material and Methods

Microorganisms

A total of 13 fungal strains belonging to the collection of Metropolitan Autonomous University (Mexico) and Research Institute for Development (France), MAU-RID, were used. The name and accession number of the used strains are presented in Table 1.

Table 1. Fungal strains from the MAU-RID collection

Accession number	Name
V12307	*Aspergillus tamarii*
Aa20	*Aspergillus niger*
C28B25	*Aspergillus niger*
V33A25	*Penicillium commune*
CINVESTAV	*Phanerochaete chrysosporium*
C23B311	Not identified
C16310	Not identified
C23308	Not identified
C17309	Not identified
C28312	Not identified
V33306	Not identified
V26316	Not identified
C11B315	Not identified

Agro-industrial material

The company Agroindustrias Unidas de México (Veracruz, Mexico) provided the CP. It was collected after the wet processing of coffee pulping, followed by sun drying for 6 days. Fragments of leaves, coffee beans and sticks were removed manually from the dry material. CP was ground in a manual mill and sieved through 12–16 mesh. Afterwards, the material was stored in dark and dry conditions until analysis.

Chemicals and reagents

Hydroxycinnamic acids (ChA, CA, *p*CA and FA) were purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). Methyl and ethyl ferulate were provided by Extrasynthese (Genay Cedex, France). All other chemicals were of analytical grade and solvents were of HPLC grade (J.T. Baker, Phillipsburg, NJ, USA).

Screening of strains

A plate assay method was used for detection of feruloyl esterase activity using a commercial enzyme (pectinase, Sigma-Aldrich) as a positive control (*14*). Plates were flooded with 1 % Bromocresol Green solution. Green/yellow zones were observed around colonies. Clearing zone formation was measured to calculate the hydrolysis index (the ratio of diameter of clearing zone to diameter of colony).

Extractable free HAs

Samples of CP (10 g) were treated with methanol or aqueous methanol at 45 °C for 20 min. Different solvents were assayed (in %): methanol 100, aqueous methanol 80 and aqueous methanol 6, all of them acidified with acetic acid 0.5. After three successive extractions with each solvent, samples were analysed by HPLC. Extractions were performed in triplicate and the final extracts were frozen and freeze-dried.

Optimisation of free HA extraction process

Extraction of free HAs from CP was carried out with the selected solvent. A CCD was performed and the design variables were extraction time (X_1: 5.9, 10, 20, 30 and 34.4 min) and extraction temperature (X_2: 33.7, 37, 45, 53 and 56.3 °C), while response variables were the extractable free HAs. The independent variables were coded to three levels: –1, 0, and +1. Responses were analysed by RSM, and the generated runs are shown in the first four columns of Table 2.

Table 2. Factorial design for the extraction of free hydroxycinnamic acids

Natural variables		Coded variables		*w*(chlorogenic acid)	*w*(caffeic acid)	*w*(*p*-coumaric acid)	*w*(ferulic acid)
Time	Temperature	X_1	X_2				
min	°C			mg/kg DCP	mg/kg DCP	mg/kg DCP	mg/kg DCP
30	53	+1	+1	81.1	8.1	2.6	2.1
30	37	+1	–1	63.2	5.7	2.1	1.2
10	53	–1	+1	62.7	6.6	1.9	1.4
10	37	–1	–1	38.4	4.6	1.7	0.9
20	45	0	0	65.7	5.8	1.8	1.1
20	56.3	0	$(2)^{1/2}$	67.6	7.6	2.8	1.9
20	33.7	0	$-(2)^{1/2}$	47.0	5.5	1.8	0.9
34.4	45	$(2)^{1/2}$	0	69.1	7.0	2.0	1.8
5.9	45	$-(2)^{1/2}$	0	40.2	4.3	1.2	0.7

DCP=dry coffee pulp

For the RSM analyses (*15*), a polynomial equation was applied as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + \varepsilon \quad /1/$$

where Y is the mass fraction (mg/kg) of four extractable free HAs; β_0 is the independent variable; β_1, β_2, β_{11}, β_{22} and β_{12} are the coefficients of the model; X_1 and X_2 are the independent variables related to the factors, and ε is the error of the model.

Alkaline hydrolysis

Hydrolysis was performed in the CP treated with aqueous methanol. Dried CP was milled and reduced to the size of 250 mesh. This material (5 g) was treated with 2 M NaOH (50 mL) for 2 h at 55 °C under stirring conditions (150 rpm). After alkaline hydrolysis, the extracts were concentrated and recovered with ethyl acetate and HAs were quantified by HPLC.

Acid hydrolysis

CP treated using alkaline hydrolysis was utilised successively with 50 mL of 6 M HCl for 1 h at 55 °C. Samples were stirred at 150 rpm. Extracts were concentrated and recovered with ethyl acetate and analysed by HPLC.

HPLC analysis

HAs were quantified by using an HPLC system (Agilent 2000, Agilent Technologies, Santa Clara, CA, USA) with a diode array. The mobile phase was water/methanol/acetic acid (80:19.9:0.1 by volume) in isocratic mode (*16*). The HPLC column Platinum C-18-EPS Rooket (53×7 mm, 3 μm, part no. 32246, CTRI Alltech Associates Inc, Deerfield, IL, USA) at a flow rate of 1 mL/min was used. Identification of compounds was achieved by comparing UV absorbance ratios with those of the standards (Sigma-Aldrich) and retention times were as follows (in min): ChA 1.4, CA 6.5, FA 11.0 and *p*CA 15.4.

Solid-state fermentation

SSF was carried out in glass columns. Each column was packed with treated CP, previously sterilised (121 °C for 15 min) and inoculated with $2 \cdot 10^7$ spores per g of dried matter. CP was impregnated with five different dilutions (0, 25, 50, 75 and 100 %) of a mineral salt solution in g per 100 g of solid substrate (dry mass) (*17*): diammonium tartarate 12.3, yeast extract 3.4, KH_2PO_4 1.3, $CaCl_2 \cdot 2H_2O$ 0.09 and $MgSO_4 \cdot 7H_2O$ 3.3; maltose 16.7 was added as a starter. Glass columns were incubated at 30 °C and aerated at a fixed rate of 20 mL per min. CO_2 production was monitored on-line as an indirect measurement of growth (*18*), while its concentration in the dry air stream outlet from the fermentation column was monitored online using a gas chromatograph (Gow-Mac 580, Bethlehem, PA, USA), equipped with a thermal conductivity detector and an automatic injector; a concentric column (CTRI Alltech Associates Inc, Deerfield, IL, USA) and an acquisition data program (Chroma Software, Biosystèmes, Dijon, France).

Enzymatic extracts

An aliquot (5 g of CP wet mass) was mixed thoroughly on a magnetic stirrer with 30 mL of MOPS buffer (3-[N-morpholino]propanesulphonic acid) 100 mM, pH=6.5. The mixture was stirred for 30 min at 4 °C. The enzymatic solution was filtered and stored at 4 °C (*17*).

Feruloyl esterase activity

Enzymatic activities were measured using ethyl and methyl ferulate as substrates. Enzymatic assays were performed in 100 mM MOPS buffer, pH=6.5, at 37 °C. Activities were expressed in nanokatal (nkat), where 1 nkat is defined as the amount of enzyme that catalyses the release of 1 nmol of cinnamic acid per s (*17*).

Data analysis

Regressions and analysis of variance (ANOVA) were performed for each hydroxycinnamic acid studied. Significance was evaluated with the Fisher test (F) at $p<0.05$, and the determination coefficients (R^2) were obtained for each extracted compound. The significance of coefficients obtained in regressions was assessed by Student's t test with $p<0.05$. Regressions and ANOVA were calculated by using the software Microsoft Excel® and response surfaces were generated by using the statistical package design experiments Fusion Pro v. 6.7.0 (S-MATRIX Corp., Eureka, CA, USA).

Results and Discussion

Extraction of free HAs

Solvent extraction of free HAs was performed for CA, FA and *p*CA, and the results were expressed as mg of HA per kg of dried CP (DCP). Extractions with 80 % aqueous methanol presented higher yields of CA ((9.8± 0.03) mg/kg of DCP) and *p*CA ((3.36±0.7) mg/kg of DCP) in comparison with 100 and 60 % methanol. In order to produce feruloyl esterases from CP, treatment with 80 % aqueous methanol could be positive because CA and *p*CA have been reported as inhibitors of these enzymes (*4*). In the case of FA, we obtained (3.28±0.03) mg/kg of DCP with 80 % methanol. The average values of the experimental results of the factorial design are shown in Table 2. Contour lines of the analysis of RSM are shown in Fig. 1, the maximum extractable free HAs were found in the range from 30 to 35 min and close to 55 °C.

Experimental data were fitted to the second-order polynomial model and the equations obtained for each HA were tested to determine the variability in the responses. Regression coefficients of the intercept, linear (time and temperature), quadratic (time^2 and temperature^2) and cross--product terms (time×temperature) are shown in Table 3. The models fitted with $R^2 \geq 0.87$ gave evidence of a high goodness of fit to the experimental data.

Extraction conditions at 56 °C for 34 min showed the best results and 76.2, 9.1, 3.1 and 2.7 mg/kg of DCP of ChA, CA, *p*CA and FA, respectively, were obtained. These values represent a small proportion of HAs in PC, which indicates that most of these compounds can be strongly linked to the cell wall. Kim *et al.* (*19*) found similar results when they studied the HAs of two varieties of wheat bran (red and white wheat bran) before phenolic acid analysis.

In Table 4, the experimental results of solvent extraction, alkaline and acid hydrolysis are shown. Data obtained at optimised conditions (56 °C, 34 min) allowed for obtaining similar results as in the RSM analysis (Fig. 1). In alkaline hydrolysis, a higher HA content of ChA

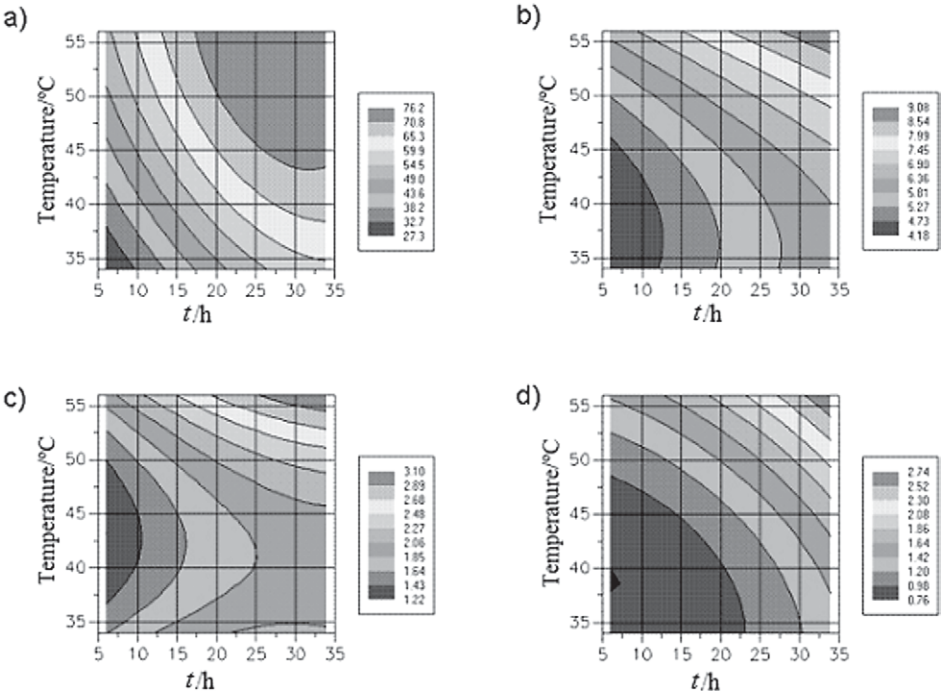

Fig. 1. Contour lines of the free hydroxycinnamic acids analysed in the study: a) chlorogenic acid, b) caffeic acid, c) *p*-coumaric acid, d) ferulic acid. Results are expressed in mg per kg of dry coffee pulp

Table 3. Regression coefficients of the predicted quadratic models to the responses of the free hydroxycinnamic acids

	Chlorogenic acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid
R^2	0.9124	0.8768	0.8871	0.9513
Intercept	0.1963*	13.0602*	10.0535*	4.6184*
Time	0.0872*	0.0576*	0.0150*	– 0.0564*
Temperature	0.1098*	– 0.5015*	– 0.4171*	– 0.1903*
Time^2	–0.0009	– 0.0004	– 0.0009	0.0008*
Temperature^2	–0.0008*	0.0067*	0.0047*	0.0024*
Time×temperature	–0.0007*	0.0009	0.0011	0.0012*

*significant ($p<0.05$)

Table 4. Hydroxycinnamic acid content in dry coffee pulp

w(hydroxycinnamic acid) / mg/kg DCP	Extractions			
	Aqueous methanol (80 %)	Alkaline hydrolysis (2 M NaOH)	Acid hydrolysis (6 M HCl)	Total
Chlorogenic acid (ChA)	84.1±4.7	1954.7±257.7	35.4±0.4	2074.2
Caffeic acid (CA)	9.0±0.6	995.1±62.7	ND	1004.1
p-Coumaric acid (pCA)	3.6±0.4	94.4±6.3	ND	98.0
Ferulic acid (FA)	3.4±0.17	108.3±7.6	2.8±0.6	114.5

ND=not detected

was observed, followed by CA, while in acid extraction, residual ChA and FA were released.

The amount of HAs reported in CP particularly varies; Labat *et al.* (*20*) reported a FA mass fraction close to 1000 mg/kg and CA around 3100–16000 mg/kg, but the extraction conditions were not described. On the other hand, Benoit *et al*. (*8*) reported 240 mg/kg of FA and 2660 mg/kg of CA in CP after alkaline hydrolysis, which represents mass fractions of 2.2 and 1.36 mg/kg higher, respectively, than those obtained in this work.

Extraction of free HAs from natural substrates is complicated because of their diversity and tendency to oxidation and hydrolysis. Different methods use preventive measures to protect the phenolic compounds or solvent extractions (*21*). RSM has been used in the extraction of HAs from different fruits (*22*). Selective extraction of extractable free HAs is required for biotechnological applications of CP. In the case of CA and *p*CA, CP could be used as a substrate for enzyme production by SSF. An improvement of the production of feruloyl esterases could be reached after the extraction of these HAs from different by-products to avoid inhibition of enzymatic activity (*4*).

Production of feruloyl esterases in SSF

A collection of 13 filamentous fungi was screened (Table 1); most of the strains belonged to the genera *Aspergillus* and *Penicillium*. The strain *Aspergillus tamarii* V12307 was selected for its ability to produce feruloyl esterase according to the results obtained for clearing zone formation. A high hydrolysis index was observed (2.5±0.2) in *A. tamarii*, while the other strains did not show any interesting enzymatic activity (<1.2). Enzymatic production was carried out in SSF by using treated CP as the substrate and mixed with different proportions of a culture medium containing maltose, which has been reported for feruloyl esterase production in sugar beet pulp (*17*). Fig. 2 shows the evolution of CO_2 production during the fermentation of *A. tamarii* V12307 with five different dilutions of culture medium.

According to the culture medium added to CP, the specific respiratory activity (μ_{CO_2}), considered as an estimation of the specific growth rate (μ), increased while germination time (t_{lag}) decreased (Table 5). This behaviour could be explained by the addition of nutrients. Panagiotou *et al*. (*23,24*) also reported the effect of medium composition and environmental parameters on the production of feruloyl esterase, xylanase and arabinofuranosidase by a *Penicillium brasilianum* strain grown on brewer's spent grain under SSF.

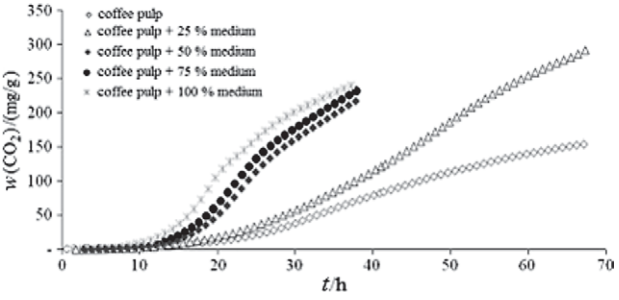

Fig. 2. CO_2 production during SSF of coffee pulp by *A. tamarii* V12307 using five different dilutions of culture medium

Table 5. Effect of different proportions of culture medium added to coffee pulp (CP)

Fermented material	μ_{CO_2}*/h^{-1}	t_{lag}*/h
CP	0.14±0.013	18.9±5.1
CP+25 % medium	0.16±0.019	17.7±4.3
CP+50 % medium	0.25±0.009	15.2±5.7
CP+75 % medium	0.26±0.014	13.3±6.9
CP+100 % medium	0.29±0.038	11.6±7.6

*p<0.0001 (univariate ANOVA)

Enzymatic activities were analysed for each culture condition at 36 h (Fig. 3). Feruloyl esterase activities were assayed with methyl and ethyl ferulate as substrates. Higher activities were observed when the culture medium was diluted 50 % for both compounds.

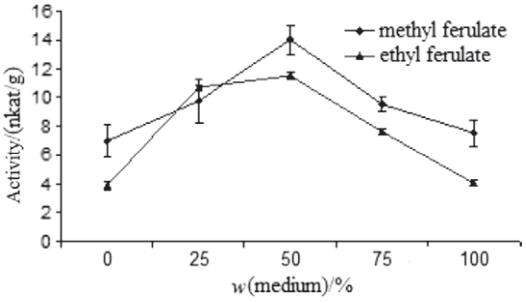

Fig. 3. Feruloyl esterase activities with different volume fractions of culture media

When untreated CP was used as the substrate and added to the culture medium diluted to 50 %, an enzyme feruloyl esterase activity of (5.95±0.003) was found as well as a μ_{CO_2} of (0.22±0.012) h^{-1}, *i.e.* the untreated CP showed lower results (58 %) in terms of feruloyl esterase production and a lower growth rate measured indirectly by CO_2 production.

A. tamarii V12307 strain produced similar feruloyl esterase activity titres as reported by Asther *et al.* (*17*) using sugar beet pulp as the substrate and an *Aspergillus niger* strain. An activity of 5 nkat per g of dry matter (DM) for methyl ferulate at 96 h of fermentation was reported by those authors, given a productivity of 1.25 nkat per g of DM per day for methyl ferulate. Enzymatic activities obtained in this work were observed at 36 h; also, the culture medium used was reduced in composition (50 %) and the observed enzyme activities were 14.0 and 10.8 nkat per g of DM for methyl and ethyl ferulate, respectively. Thus, the productivities were 9.3 and 7.2 nkat per g of DM per day, respectively.

Using *P. brasilianum*, higher productivity of 26 nkat per g of DM per day for methyl ferulate in SSF has been reported (*23*). In Fig. 4, the online monitoring of CO_2 of the selected conditions is presented. The respiratory activity rate (μ_{CO_2}) was (0.25±0.011) h^{-1} and the lag phase (t_{lag}) was (14.3±2.2) h. Also, in the same figure, the enzyme production during fermentation using ethyl ferulate as the substrate is presented. At 36 h, it was observed that higher activity was found in the stationary phase.

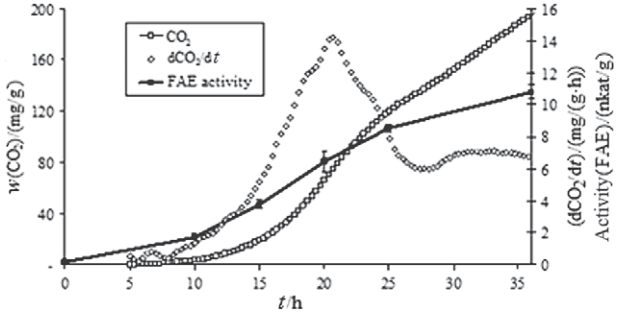

Fig. 4. CO_2 production by *A. tamarii* V12307 with the selected medium and feruloyl esterase production in solid-state fermentation of coffee pulp

There are many reports describing the properties of HAs and their conjugates; potential health benefits are observed in diets rich in fruits, vegetables and fibre (*25,26*). HAs are important components of plant cell walls. This has generated commercial interest, for example, for improving the quality of raw materials, reducing the cost of processing, increasing yields or producing functional foods (*25*). Various methods have been explored for the release of HAs from their ester-linked compounds for industrial applications.

Conclusions

Feruloyl esterase activity was produced by *Aspergillus tamarii* V12307 by solid-state fermentation in coffee pulp. Solid-state fermentation of CP allowed for the production of enzyme crude extracts with feruloyl esterase activity (14.0 and 10.8 nkat per g of dried matter per day when methyl and ethyl ferulate were used as substrates). The best results were obtained using a culture medium diluted to 50 % and incubated at 30 °C for 36 h. An improvement in feruloyl esterase production was obtained by the treatment of CP, and RSM analysis on the extraction of free HAs allowed for optimising the conditions (aqueous methanol 80:20 at 56 °C for 34 min). Alkaline and acid hydrolysis demonstrated that most of the HAs are linked to polysaccharides. The use of CP with HAs bound to the cell wall and without free HAs possibly reduces the problems with the inhibition of activity. Finally, the use of cheap substrates as carbon and energy sources and solid supports gives an interesting way of improving the value of agricultural by-products such as CP. It is a good alternative to obtain compounds with high added value such as the hydroxycinnamic acids.

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