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Effects of different solid state fermentation substrate on biochemical properties of cutinase from *Fusarium* sp.

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ABSTRACT

The present results demonstrate that the catalytic characteristics of cutinase produced by the same strain differ depending on the culture medium used. This conclusion was possible after the study of biochemical characterization and enantioselective properties of cutinases produced by *Fusarium oxysporum* in four different culture mediums. The mediums were composed of wheat bran, soybean rind, rice bran and *Jatropha curcas* seed cake, different Brazilian agricultural by-products. The largest difference can be observed on cutinase produced by *J. curcas* seed cake. This enzyme has been activated in most metal ions tested and exhibited excellent stability in organic solvent, especially hexane. The cutinase produced in rice bran showed greatest activity in the presence of *p*-nitrophenyl butyrate as a substrate, whereas the other enzymes showed greatest activity in the presence of *p*-nitrophenyl caprilate. Regarding enantioselective properties the cutinase produced in soybean rind showed the best result compared to enzymes produced in wheat bran.

1. Introduction

Cutinase (E.C. 3.1.1.74) is an enzyme that catalyzes the hydrolysis of esters bonds in cutin. Cutin is a biopolymer insoluble in water composed of fatty acids C_{16} and C_{18} and found in the outside surface of the plants' aerial parts. Cutinases are also able to hydrolyse a large variety of synthetic esters and show activity on short and long chains of triacylglicerols [1].

Studies have been conducted with the aim of biochemically characterizing the cutinases produced by different fermentation conditions, microorganisms and medium compositions. Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented products may be used directly as enzyme source [2]. A major advantage of microbial enzymes is that it shows different biochemical characteristics depending on the genetic variability of the specie. In addition, previous work performed in our laboratory demonstrates that the enzymes produced from the same microorganism and different culture medium, such as from by-products or residues rich in carbon sources do not necessarily presented the same biochemical properties. The use of different mediums for the production of enzymes can lead to the formation of isoforms with different catalytic characteristics [3]. The study of these differences, as well as of the factors that affect the activity of the enzymes is important in order to understand the action of such enzymes and the alterations they go through in different conditions of storage and use. Cutinases have been presented as a versatile enzyme showing several noteworthy properties for application in industrial products and processes [4]. Finding and characterizing new cutinases allow further expansion of the possible applications of such enzymes. The objective of this study was to biochemically characterize and compare cutinases produced by *Fusarium oxysporum* in four different culture medium composed of wheat bran, soybean rind, rice bran and *Jatropha curcas* seed cake and evaluate the differences of these enzymes in the resolution of racemic mixtures.

2. Materials and methods

2.1. Materials

p-Nitrophenyl butyrate (*p*-NPB) was purchased from Sigma–Aldrich Brazil Co. (São Paulo, SP, Brazil). Triton X-100, tetrahydrofuran and other reagents were purchased from Merck (São Paulo, SP, Brazil). The agricultural by-products used as the solid culture mediums were donated by different companies from Campinas, SP, Brazil.

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2.2. Microorganism preservation and preparation of the pre-inoculums

A *F. oxysporum* strain isolated from soil and plants was selected in a previous study with 400 strains of fungi as the best cutinase producer. The culture medium used for this previous selection contained apple cutin as the sole carbon source [5]. The strain was maintained in potato dextrose Agar (PDA; Acumedia Manufactures Inc. Lansing, MI, USA) slants and stored at 4 °C. The pre-inoculum was prepared by adding 2.5 mL of distilled water to remove the spores, obtaining a suspension containing 7×10^7 spores/mL.

2.3. Cutinase production

Three by-products were selected due to cutinase activity in preliminary experiments and good development of *F. oxysporum* strain: wheat bran, soybean rind and rice bran [3]. *J. curcas* seed cake was also included in this study, because of its availability. Brazil is a major producer of oil and the use in biotechnological process of cake produced after extraction is very advantageous from the environmental and economic point of views [6]. Crude enzymes extracts were prepared as previously reported [3]. The crude cutinases produced in each medium (wheat bran, soybean rind, rice bran and *J. curcas* seed cake) were used in all experiments of the biochemical characterization. The crude cutinases produced in the mediums with wheat bran and soybean rind were used for the enantioselective characterization.

2.4. Cutinase assay

The activity against *p*-nitrophenyl butyrate (*p*-NPB) was determined as previously reported [5]. The hydrolysis of *p*-NPB was spectrophotometrically monitored for the formation of *p*-nitrophenol at 405 nm.

One unit of cutinase activity was defined as the amount of enzymes required to convert 1 μmol of *p*-NPB to *p*-nitrophenol per minute, under the specified conditions [7].

2.5. Biochemical characteristics of the crude cutinases

2.5.1. Effects of temperature and thermal stability

To determine the optimum temperature for cutinolytic activity, enzymatic reactions at various temperatures over the range of 25–75 °C were performed by using the procedure described above (cutinase assay).

In order to determine the thermal stability, aliquots of each crude cutinase in Eppendorf tubes were incubated for 1 h at various temperatures over 30 °C to boiling temperature. After incubation, the tubes were rapidly cooled in an ice bath and then brought to room temperature. The activities were determined with the cutinase assay described previously. The percentage residual activities were calculated by comparison to untreated control enzyme. All the above tests were carried out in duplicate.

2.5.2. Effects of pH and pH stability

The effect of pH on cutinolytics activities was determined by using the following buffers (all at 0.1 mM): acetate buffer pH 3.6, 4.0, 5.0, 5.6; phosphate buffer pH 6.0, 6.5, 7.0; Tris–HCl buffer pH 8.0, 8.5, 9.0 and borate–NaOH pH 9.5, 10.0. The activities were determined with the cutinase assay described previously at optimum temperature of the enzymes.

The same buffers were used to determine pH stability of the crudes cutinases for cutinolytics activities. The mixture of each crude cutinases and 1 mL of buffer given above was incubated for 24 h at optimum temperature of the enzymes. The activities were determined with the cutinase assay described previously. The percentage residual activities were calculated by comparison to untreated control enzyme. All the above tests were carried out in duplicate.

2.5.3. Effect of metal ions

The effects of $CaCl_2$, KCl, $HgCl_2$, $MnCl_2$, $CoCl_2$, K_2HPO_4, $NaNO_3$, $FeSO_4$, $MgSO_4$, $ZnSO_4$, $MnSO_4$, K_2SO_4, Na_2SO_4 and $NaHSO_3$ on the cutinolytic activity were investigated. Final concentrations of each metal ion in the reaction mixture were 1 mM and 10 mM. The percentages of activities were determined by comparison with the control mixture with no metal ion added. All the above tests were carried out in duplicate.

2.5.4. Substrate specificity

The influence of different substrates on the cutinolytic activity was investigated replacing the *p*-NPB of the reaction described above (cutinase assay) by the following substrates: *p*-nitrophenyl caprilate (p-NPC), *p*-nitrophenyl laurate (p-NPL) and *p*-nitrophenyl palmitate (*p*-NPP). All the above tests were carried out in duplicate.

2.5.5. Stability in organic solvent

The stability of the crude cutinases in acetone, methanol, ethanol, butanol, propanol, hexane and octanol was tested. The mixture of crude cutinases and 1 mL of each organic solvent given above was incubated for 1 h at optimum temperature of the enzymes. After this the organic solvents were dried with N_2 and the activities were determined by comparison with the control mixture with no organic solvent added. All the above tests were carried out in duplicate.

2.6. Esterification of (R) and (S)-2-octanol and enantioselective esterification of (R,S)-2-octanol

The reactions of esterification and enantioselective esterification were performed with the crude cutinase produced in mediums composed by wheat bran and soybean rind, these enzymes were selected after preliminary tests (thin layer chromatography), that indicated these enzymes were the most enantioselective (data not shown).

2.6.1. Esterification of (R) and (S)-2-octanol

The esterification of (*R*) and (*S*)-2-octanol was performed in a mixed reaction containing 40 mM of each enantiomerically pure of 2-octanol, 40 mM of octanoic or hexanoic acid, 4 mL of hexane and the crude cutinase of wheat bran and soybean rind. The reaction was carried out at 30 °C, 168 h and 130 rpm. Experiments with no added enzyme were carried out to evaluate the percentage of spontaneous esterification in the system. The ester formation was followed using a Chrompack gas cromatograph (Chrompack Co., Holland) equipped with a flame ionization detector and a column (i.d. 0.32 mm, length 30 m) CP-WAX 52 CB. The initial temperature of the column was 50 °C for 2 min then the temperature increase at a rate of 10 °C/min up to 220 °C. For the analysis, H_2 was used as the carrier gas and the detector and injector were set at 250 °C and 220 °C, respectively.

2.6.2. Gas chromatography analysis

The reaction conditions of the resolution of (*R,S*)-2-octanol was performed under the same conditions described above (esterification of (*R*) and (*S*)-2-octanol). The enantiomeric excess (ee_p) of the ester produced was determined using Chrompack gas chromatograph (Chrompack Co., Holland) equipped with a fused silica capillary quiral column BETA DEX™ 120 (0.25 mm, 60 m, Supelco). The initial temperature of the column was 95 °C for 30 min, and then the temperature increased at a rate of 5 °C/min up to 220 °C.

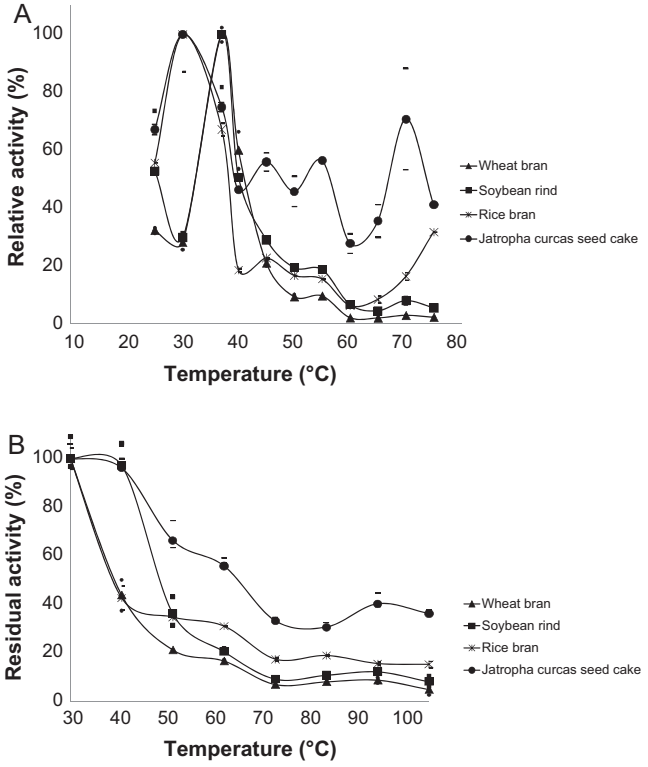

Fig. 1. Optima (A) and stability (B) temperature value for crude cutinase produced by culture mediums compounded by wheat bran, soybean rind, rice bran and *Jatropha curcas* seed cake.

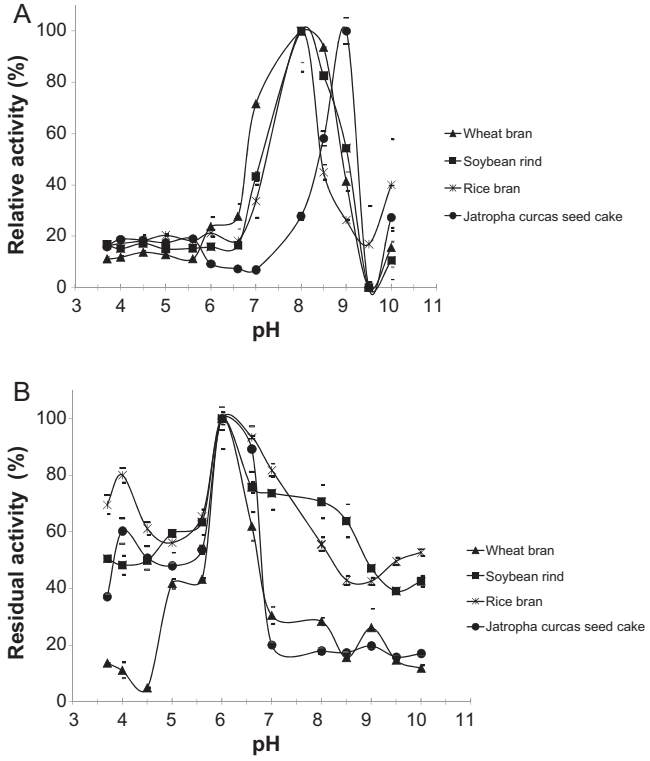

Fig. 2. Optima (A) and stability (B) pH values for crude cutinase produced by culture mediums compounded by wheat bran, soybean rind, rice bran and *Jatropha curcas* seed cake.

For the analysis, H_2 was used as the carrier gas and the detector and injector were set at 250 °C and 220 °C, respectively.

3. Results and discussion

The results of biochemical characterization of crude cutinases produced in different culture mediums are presented below. It was possible to confirm the catalytic specificities of these enzymes according to the medium used.

3.1. Biochemical characteristics of the crude enzymes

3.1.1. Effects of temperature and thermal stability

Optimum temperature of crude cutinases produced on mediums composed of rice bran (8.5 U/mL) and *J. curcas* seed cake (9.0 U/mL) was 30 °C, while enzymes produced on mediums with wheat bran (24.0 U/mL) and soybean rind (21.0 U/mL), showed optimal temperature at 37 °C. The other peaks found in the enzyme produced in *J. curcas* seed cake may indicate the presence of isoforms of cutinase or other enzymes present on crude extract that also hydrolyze *p*-NPB (Fig. 1A). These results are in accordance with earlier reports in which the temperature optimums of two cutinases from *Fusarium solani* are between 35 and 45 °C [8].

The enzymes produced on mediums composed of wheat bran and rice bran were completely stable at 30 °C when incubated for 1 h. The enzyme produced on medium composed of soybean rind remained completely stable at temperatures 30 and 40 °C, the same stability was observed in the enzyme produced in the medium composed of *J. curcas* seed cake. This enzyme exhibited superior thermostability, with residual activity of over 60% after 1 h at 50 °C (Fig. 1B). Earlier results reported indicated that cutinase from *F. solani* pisi was stable from 40 to 60 °C [9].

3.1.2. Effects of pH and pH stability

As shown in Fig. 2A, the pH optima of crude cutinases produced on mediums composed of wheat bran (28.0 U/mL), soybean rind (25.0 U/mL) and rice bran (11.0 U/mL) were found to be 8.0, whereas the crude cutinase produced on medium with *J. curcas* seed cake was found to be 9.0 (11.0 U/mL). Similar results were reported earlier for pH optima of cutinases [10,11]. The results confirm the literature values, which indicate that most fungal and bacterial cutinases presented optimum pH in the range of 9.0 [12].

Crude cutinase produced in medium composed of rice bran was stable in the pH range of 6.0–7.0 after 24 h, retaining more than 80% of the initial activity. This enzyme was also more stable at acid pH, with over 60% of residual activity after 24 h in pH ranging from 3.5 to 5.5. The crude cutinase of soybean rind retained more than 70% of the initial activity from pH 6.0 to 8.5 after 24 h. The crude cutinase of *J. curcas* seed cake showed pH-stability ranging from pH 6.0 to 6.6 after 24 h. The enzyme produced in medium composed of wheat bran, was stable at pH 6.0 and relatively stable at pH 6.6 (Fig. 2B). The stability of enzymes in acid, neutral and basic pH is important in terms of industrial application. These results are in accordance with earlier reports in which the pH-stability of cutinase from *F. solani* pisi was between 6.0 and 8.5 [8].

3.1.3. Effect of some metal ions

To determine whether the cutinase requires a metal cofactor for activity and if the need of a cofactor changes according to the culture medium of the enzyme, they were incubated with the metal ions and then assayed for cutinase activity against *p*-NPB. The values of enzymatic activity of the controls were: 32.0 U/mL (wheat bran), 24.0 U/mL (soybean rind), 15.0 U/mL (rice bran) and 9.5 U/mL (*J. curcas* seed cake). The results in Fig. 3 indicates that the enzyme produced on mediums composed with soybean rind and rice bran were inhibited or slightly activated in the presence of the metal ions tested. The enzyme produced in medium with wheat bran was

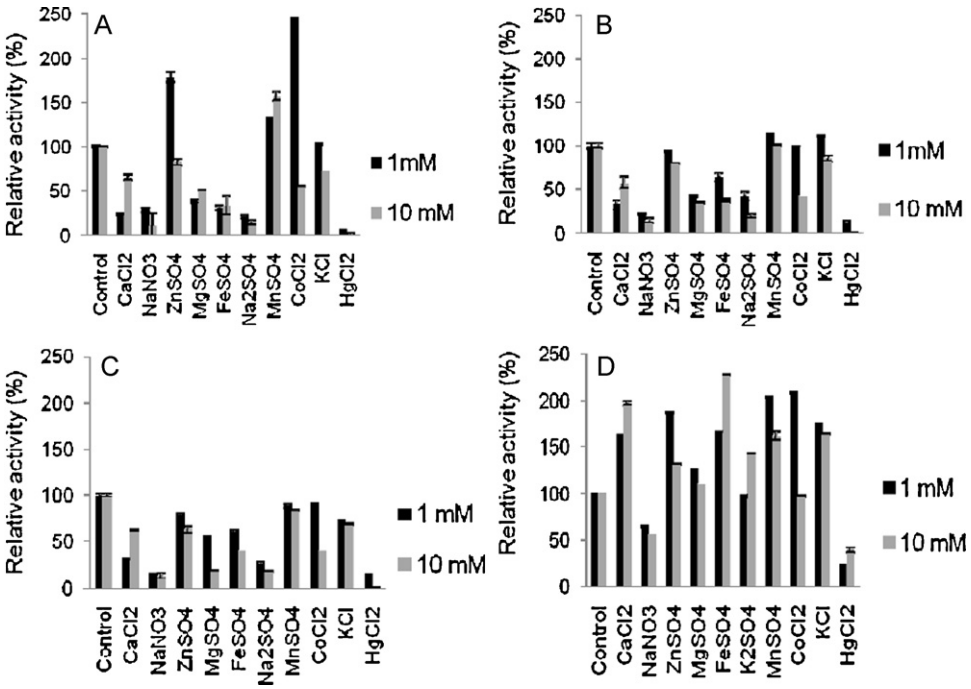

Fig. 3. Effect of metal ions (1 and 10 mM) on the activity of cutinases produced by culture mediums compounded by wheat bran (A), soybean rind (B), rice bran (C) and *Jatropha curcas* seed cake (D).

mostly activated in the presence of $ZnSO_4$ and $CoCl_2$ in concentration of 1 mM. The enzyme produced in medium with *J. curcas* seed cake, different of the other enzymes, was activated in the presence of most metal ions, highlighting MnSO4, $CaCl_2$, ZnSO4, $FeSO_4$, $MnSO_4$, $CoCl_2$ and KCl (1 mM) and $FeSO_4$ (10 mM). The enzymes were quite inhibited in the presence of $HgCl_2$ in both concentrations, indicating that –SH groups are essential for the enzyme activity [13].

The distinct behavior regarding the need for minerals in the enzyme produced in *J. curcas* seed cake can be attributed to changes in the protein structure of this enzyme and consequent change in the active site. Other possible explanations can be attributed to the binding of minerals to some inhibitor present in the crude extract or even due to its own medium, which may be deficient in these minerals. Previous research has shown that esterase and cutinases were similar to the enzymes produced in wheat bran, soybean rind and rice bran, the enzymes are inhibited or lightly activated by minerals tested [14–16]. Cutinase activated by the presence of minerals were not found in previous studies, nor were they found with the enzyme produced in *J. curcas* seed cake. This feature can be exploited in industrial processes in which these minerals are necessary.

3.1.4. Substrate specificity

The chain length specificity of esters was investigated using *p*-nitrophenyl-fatty acyl esters (Fig. 4). The enzymes produced in wheat bran (54.0 U/mL), soybean rind (34.0 U/mL) and *J. curcas* seed cake (25.0 U/mL) had higher specificity in medium-chain fatty acids (*p*-NPC). The enzyme produced in medium composed of rice bran (10 U/mL) showed higher specificity in short-chain fatty acids (*p*-NPB). You can see that the four enzymes had higher specificity for short and medium chain fatty acids and the activities toward long chain *p*-NP-fatty acids were low. Other studies have indicated that cutinases showed specificity for short and medium carbon chain of fatty acids [8,14,17].

3.1.5. Stability in organic solvent

In order to evaluate the stability in organic solvents the samples were compared with controls, with activity values of 24.0 U/mL for the enzyme produced in wheat bran, 21.0 U/mL for the enzyme produced in soybean rind, 11.0 U/mL for the enzyme produced in rice bran and 9 U/mL for the enzyme produced in *J. curcas* seed cake. The results in Fig. 5 indicate that the enzymes produced in mediums composed with wheat bran, soybean rind and rice bran were not very stable in organic solvents. The enzyme of soybean rind and rice bran were relatively more stable in the presence of hexane, keeping 71% and 66% respectively of their activities compared with control. On the other hand, the enzyme produced in medium with *J. curcas* seed cake was fairly stable in the presence of organic solvents, its activity remained high in all solvents evaluated, highlighting the hexane, butanol and propanol. The results suggest that the enzyme of *J. curcas* seed cake is stable in the presence of organic solvents, which is a very interesting property for industrial applications. The use of organic solvents in biocatalysis reactions has some advantages such as increased solubility of organic substrates insoluble in water and the ability to shift the thermodynamic equilibrium of many processes for the synthesis path [18]. As an example, we can mention the reactions of esterification and transesterification for the production of flavorings [19,20], production of biodiesel [21] and the resolution of racemic mixtures [3].

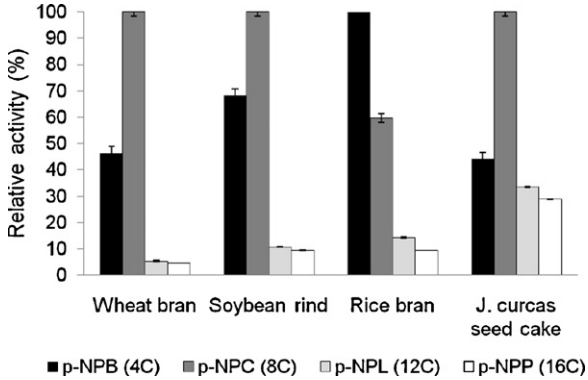

Fig. 4. Substrate specificities of crude cutinase produced in different culture mediums toward *p*-nitrophenyl esters.

Table 1
Esterification of pure alcohols (*R*) and (*S*)-2-octanol with octanoic and hexanoic acids catalyzed by crude cutinases produced in the culture mediums compounded by wheat bran and soybean rind.

Substrate for production of cutinase	% Esterification			
	Octanoic acid		Hexanoic acid	
	(*R*)-2-octanol	(*S*)-2-octanol	(*R*)-2-octanol	(*S*)-2-octanol
Wheat bran	15.4	45.4	8.5	7.8
Soybean bran	26.3	42.5	4.8	6.9

Table 2
Conversion (*c*) and enantiomeric excess of *S*-ester product (ee_p) after 168 h of reaction of racemic alcohol (*R,S*)-2-octanol with octanoic and hexanoic acids catalyzed by crude cutinase produced in the culture mediums compounded by wheat bran and soybean rind.

Substrate for production of cutinase	Octanoic acid			Hexanoic acid		
	c (%)	ee_p (%)	*E*	*c* (%)	ee_p (%)	*E*
Wheat bran	38.3	45.6	3.5	6.8	25.7	1.7
Soybean bran	40.4	59.8	5.9	7.8	23.8	1.7

c: conversion given as the percentage of initial alcohol esterified after the reaction time; ee_p enantiomeric excess of the ester produced; *E*: enantiomeric ratio.

3.2. Esterification of (R) and (S)-2-octanol and enantioselective esterification of (R,S)-2-octanol

3.2.1. Esterification of (R) and (S)-2-octanol

Table 1 presents the values of esterification of enantiomerically pure alcohols (*R*) and (*S*)-2-octanol with octanoic and hexanoic acids catalyzed by crude cutinases produced in mediums composed with wheat bran and soybean rind. The results obtained using the octanoic acid show good rate of esterification for both enzymes, indicating enantiopreference for the (*S*)-enantiomer. The results obtained using hexanoic acid show low yields for both enzymes after 168 h of reaction, and it is also difficult to ascertain any preference for one of the enantiomers. Although octanoic and hexanoic acids are similar in number of carbons, the results show a greater presence in the esterification of octanoic acid. A possible explanation may be due to the fact that the active site of each enzyme recognizes more efficiently the carbon chain of octanoic acid.

3.2.2. Enantioselective esterification of (R,S)–2-octanol

In Table 2 the results of conversion (*c*), enantiomeric excess of *S*-ester product (ee_p) and enantiomeric rate (*E*) after 168 h of reaction of racemic alcohol (*R,S*)-2-octanol with octanoic and hexanoic acids catalyzed by crude cutinase produced in mediums composed with wheat bran and soybean rind are presented. The results obtained using the octanoic acid show that the enzyme produced in soybean rind was more enantioselective. Since this enzyme has good values of conversion, enantiomeric excess and enantiomeric rate, its use as a catalyst in the resolution of racemic alcohols such as (*R,S*)-2-octanol, appears to be quite interesting. The results obtained using hexanoic acid in relation to the values of conversion, enantiomeric excess and enantiomeric rate were lower than the reaction with octanoic acid. The results obtained using the octanoic acid showed to be promising. The optimization of enantioselective reactions may facilitate the achievement of the most interesting results, allowing the use of these enzymes especially that produced in soybean rind, for the enantioselective process.

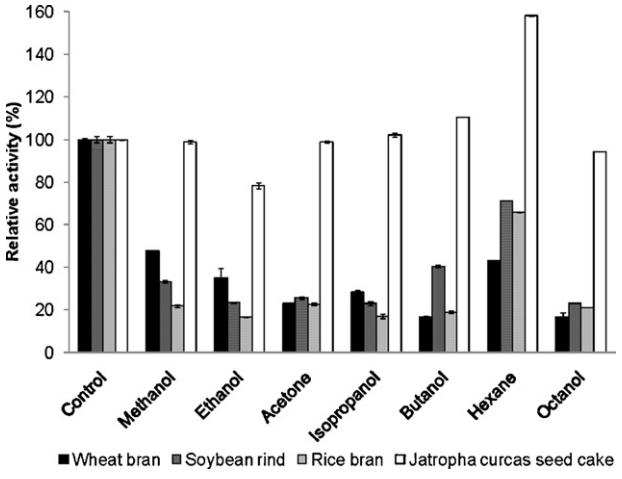

Fig. 5. Effect of different organic solvents on the activity of crude cutinase.

4. Conclusions

The results confirm that although the enzyme has been produced by the same microorganism, its catalytic characteristics differ depending on the medium used. One possible explanation for this phenomenon is that the use of different mediums for the growth of microorganism forces it to adapt to these different sources of nutrients, thus producing isoforms. Another possible explanation is that possible components of the medium stabiles in a different way the enzyme.

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