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Production of lignocellulosic enzymes by Streptomyces thermocerradoensis I3 in unconventional culture medium

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ABSTRACT:The present work aimed to contribute with new data about the production of lignocellulolytic enzymes produced by Streptomycesthermocerradoensis I3, isolated from Brazilian central savannah soil (also known as Cerrado), using the rhizome of two plants from the Cerrado, Cochlospermumregium (CR) and Jatropha elliptica (JE), as well as wheat bran (WB) as a culture medium. In addition, verify the influence of pH on the enzymes production using WB and evaluate the effectiveness of enzymatic extract produced in CR and JE flours. The higher CMCase activity detected occur in WB (6.07 ± 0.13 IU mL^{-1}) followed by JE (4.18 ± 0.23 IU mL^{-1}), while it was not detected in CR. The higher xylanase activity detected was 4.14 ± 0.10 IU mL^{-1} in WB, followed by 2.13 ± 0.14 IU mL^{-1} in CR and was not detected in JE. Regarding the influence of pH on the activity of CMCase in WB, was observed that the highest activity occurred at pH 7.5 (13.22 ± 0.26 IU mL^{-1}); and for the activity of Xylanases, it was observed a higher activity at neutral pH (4.03 ± 0.20 IU mL^{-1}).The enzymatic hydrolysis of the CR and JE flours, also promoted the increase of about 30% of phenolic compounds and total soluble sugars, with 48 h of exposure to enzymatic extract, which demonstrated that the enzymatic extract was efficient. This fact suggests the use of this extract for hydrolysis for enrichment food.

KEYWORDS: Semi-solid fermentation; Enzymatic saccharification; Cellulases; Xylanases

I. INTRODUCTION

Enzymes are dynamic biocatalysts that because of their highly specific nature, have a wide range of applications in the industrial sector (1). Hydrolytic enzymes are useful tools in the production of food, medicines, textiles, animal feedstuffs, baking, brewing, paper, and biofuels, through the transformation of lignocellulosic biomass into products with high added value. Some enzymes as cellulases, amylases, xylanases, lipases, proteases and others have been produced by microorganisms isolated from soil, water, plants and decomposing lignocellulosic biomass (2, 3).

Cellulases hydrolyse β−1,4-d-glucan linkages in the cellulose structure, releasing glucose, cellobiose and cello-oligosaccharides. They constitute the most comprehensively studied enzymatic complex including endo-glucanases (EG; EC 3.2.1.4), cellobiohydrolases (CBH; EC 3.2.1.91) and β-glucosidases (BGL; EC 3.2.1.21) (4). Xylanases hydrolyse xylan, the main hemi-cellulosic polysaccharide, and when associated to other hemicelluloses components binds to the surface of cellulose microfibrils by hydrogen bonding and hinders cellulase action during saccharification (5). In order to improve the applicability of cellulases and xylanases it is sought to improve characteristics necessary for the industries, such as catalytic efficiency in insoluble cellulosic substrates, thermostability, stability to a certain pH, greater tolerance to the inhibition by the final product and multifunctional nature increasing the catalytic efficiency (6). Although many enzymes with industrial applicability are derived from fungi, the isolation and characterization of new enzymes from bacteria has been evaluated since heterologous production, high specific activity and less stringent pH requirements of bacterial systems can occur (7).

Some Streptomyces strains have been isolated from the soil of the Brazilian central savannah, known as Cerrado (8). The Cerrado is

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the second largest biome in Latin America, with more than 200 million hectares, covering 22% of the Brazilian territory. In spite of its immense biodiversity and the detailed characterization of the flora and fauna in the Cerrado biome, little is still known about the bacterial communities of the soil (9, 10). Several strains of Streptomyces form the Cerrado soil showed the ability to produce large amounts of endoglucanases and cellobiohydrolases (11-14) as S.thermocerradoensis bacteria I3. In addition, in Cerrado biodiversity there are several plant species that can be used as culture media, such as Cochlospermum regium and Jatropha elliptica (Pohl), which are also popular in folk medicine because of their high levels of phenolic compounds and antioxidant activity.Moreover, the rhizomes of these plants can be used to produce flours (15-17). Enzymatic treatment of flours has also shown significant potential for improving the nutritional quality and health effects of both foods and ingredients (18)

Since there are few reports in the scientific literature about the production of hydrolytic enzymes by S.thermocerradoensis, the present work aimed to contribute with new data on the production of lignocellulolytic enzymes by Streptomyces thermocerradoensis I3 using the rhizome of two native plants from the Brazilian's Cerrado, as well as wheat bran as substrate. In addition, verify the influence of pH on enzyme production using WB and evaluate the effectiveness of enzymatic extract produced, by enzymatic hydrolyse of JE and CR flours.

II. MATERIAL AND METHODS

Microorganisms

A strain of Streptomyces thermocerradoensis I3 was obtained from the Cerrado soil and is stored at Microorganisms Bank of the Laboratory of Biochemistry and Genetic Engineering - ICB - UFG. For obtention of the strain of Streptomyces thermocerradoensis I3 the sugarcane bagasse (SCB) was dried in the environment, being exposed to the bacterial community of Cerrado soil. Five grams of dried SCB was washed with 10 mL of saline solution (NaCl, 0.9 %) and centrifuged (3,000 rpm for 10 min), and the supernatant was diluted 1,000-fold with sterile saline. Aliquots of 100 µL were plated on minimal medium supplemented carboxymethylcellulose (CMC) 0.5 % (w/v) and incubated at 45 ℃ for 4 days. After the incubation, the plates were stained with Congo red (19). A strain of Streptomyces thermocerradoensis named isolated three (I3) was used to produce the enzymatic extracts. For obtention of the spores of Streptomyces thermocerradoensis I3, these microorganism were cultured on the bacterial culture dish and distributed into five colonies on Pridham medium (20) (4 $g.L^{-1}$; Malt extract 10 $g.L^{-1}$; sucrose 4.$g.L^{-1}$ and agar 20 $g.L^{-1}$), for 3 days in an oven at 37 ± 1 °C. A plate was removed and 10 ml of autoclaved water was added and the spores were obtained using a glass handle.

Substrate processing

The roots of Cochlospermum regium (CR) and Jatrhopha elliptica (JE) were collected in the municipality of Uruaçú-GO (-14.264977 S, -48.973811 W), in a region of Cerrado native forest, in July 2016. The roots were stored in low density polyethylene bags and maintained under refrigeration (6 ± 1 °C) during transportation and storage until processing took place. The roots of CR and JE were washed and sanitized with sodium hypochlorite solution at 200 ppm for 15 min, peeled and sliced manually, and allowed to dry in an air circulation oven at 35 ℃ for 48 h. The dehydrated material was milled with "Croton" type knife mill (Marconi, MA580, Piracicaba, Brazil), with a 2.0 mm diameter mesh sieve to obtain the flour.

Wheat bran (WB) was purchased in a current market of Goiania city. WB was washed three times to reduce the concentration of sugars.

Culture media and enzymatic extract preparation

Streptomyces thermocerradoensis I3 spores were inoculated into a conic flask containing 15 mL of pre-autoclaved minimal medium (Na_2HPO_4 - 7.0 $g{\cdot}L^{-1}$; K_2HPO_4 - 3 $g{\cdot}L^{-1}$ NaCl - 0.5 $g{\cdot}L^{-1}$; NH_4Cl - 1.0 $g{\cdot}L^{-1}$) and 5 g of WB, CR or JE substrate, separately. At the end of a 5-day incubation period at 37 ± 1°C, 50 mL of water and 50 mL of Tween-80 (0.1 $g{\cdot}100g^{-1}$) were added, centrifuged at 7500 RPM for 15 min at 4 ± 1°C and the supernatant stored frozen (-20 ± 1°C) until the assays were carried out.

Enzymatic Assays

The enzymatic extract was evaluated for the activities of xylanase and CMCase (carboxymethylcellulase). The endoglucanase activity was evaluated using CMC in a micro assay according to Ramada, Lopes (21) . The xylanase activity was determined using 1% (w/v) bechwood xylan solutions as substrate. The activity was determined in a micro assay by adding 10 µL of sample to 90 µL of the 1% xylan solution (22). The solution was incubated at 50 ± 1 ℃ for 5 min followed by quantification of the reducing sugars

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by the ADNS method (23). A standard curve for xylose was prepared at concentrations from 0.3 to 4.2 mg.mL^{-1}. The results were presented as international units (IU) per mL of extract. One activity unit (IU) corresponded to 1 µM of glucose (CMCase) or xylan (xylanase) released per minute.

Total phenolics compounds and antioxidant activity in the flours

The flours of CR and JE were evaluated to verify the total phenolic compounds. The phenolic compounds of the flour were extracted with methanol-water solutions (80:20). The same extract was used for determining antioxidant activity. The total phenolic compound content was determined using the Folin-Ciocalteau reagent and a spectrophotometer at 740 nm wavelength. The data were expressed as milliequivalents of gallic acid per 100g of sample (24). The antioxidant capacity of the flour extract was determined by the DPPH method (25), based on the capture of the DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical by antioxidants, producing a decrease in absorbance at 517 nm wavelength.

Determination of Optimal pH

The optimal pH determination was carried out using 50 mM of sodium phosphate buffers solutions ranging from pH 6.5, 7.0 and 7.5 as described by Gama, Brito-Cunha (26). After checking the pH of the media, the procedures described in Culture media and enzymatic extract preparation and Enzymatic Assays were reproduced in three conic flasks for each pH range.

Enzymatic Hydrolysis in Jatrhopha ellipticaandCochlospermum regiumflour

In the assays for enzymatic hydrolysis, solutions of 1% (w/v) of the flour (CR and JE, separately) and phosphate buffer pH 6.0 (pH was corrected to 6.0 using NaOH or HCl 0.01N solutions) were added with 0.1 mL of enzymatic extract (3 IU·mL^{-1} for CMCase and 9 IU·mL^{-1} for xylanase, preliminary test as described in enzymatic assay topic) previously produced using WB as substrate. The reaction mixtures were incubated under stirring at 50° C for periods of 4, 24 and 48 h. As a control, flour solutions were used without the addition of the enzyme extract. The hydrolyzed and control solutions were evaluated for the reducing and total sugars by the method proposed by Miller (23), phenolic compounds according to Singleton, Orthofer (27) and the protein assays were performed according to Bradford (28). The amount of total protein was determined by absorbance reading in a spectrophotometer at 595 nm wavelength. The calibration curve was determined using Bovine Serum Albumin (BSA, Sigma®).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of three replicates. Significant differences between the means of the parameters were calculated with a one-way analysis of variance (ANOVA) using the software Graph pad prism 5®.

III. RESULTS AND DISCUSSION

Enzymatic extracts evaluation

The measured CMCase activity was 6.07 ± 0.13 IU·mL^{-1} in WB substrate, 4.18 ± 0.23 IU·mL^{-1} in JE, while it was not detected in CR. The xylanase activity was 4.14 ± 0.10 IU·mL^{-1} in WB; 2.13 ± 0.14 IU·mL^{-1} in CR and was not detected in JE (Figure 1). Only the enzymatic extract produced using WB as substrate presented both CMCase and xylanase activity. A recently research also observed that use of WB as culture media was effectively for production of cellulolytic enzymatic extracts using Streptomyces thermocerradoensis I3 (26).

The high availability of carbohydrates in CR (83.99 g.100g^{-1}) (29) and JE (about 77 g.100g^{-1}) (17), can induced the inhibition of the production of the enzymes xylanase and CMCase by this microorganism (30), which may explain the low CMCase or xylanase activity of the enzymatic extract obtained with these substrate.

The JE flour (methanolic extract) showed a content of phenolic compounds of 11.47 ± 0.87 mg eq gallic acid.g^{-1} while this value in CR flour was 8.32 ± 0.45 mg eq gallic acid.g^{-1} (Table 1).Vaher, Matso (31) determined phenolic compounds concentration in wheat bran as 1.258 mg eq gallic acid.g^{-1}, which means lower values than those found in methanolic extract obtained from Cochlospermum regium and Jatropha elliptica flours. In addition, the phenolic compounds can bind to the proteins and may deactivate the cellulolytic enzymes during cellulose hydrolysis (32-34). Phenolic compounds significantly increased the inhibition of laccase (ligninolytic) activity in Botryosphaeria rhodian (35).

Another factor that may have negatively interfered is the higher content of phenolic compounds in JE and CR flours or the distinct types of phenolic compounds compared to WB, resulting in higher enzyme production in WB treatment.Consequently, WB proved to be the best substrate to produce cellulases and xylanases by Streptomyces thermocerradoensis I3, probably because of the content of arabinoxylan in aleurone

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(36). Moreover, the aleurone layer is particularly rich in nutrients and minerals such as iron, magnesium, zinc and calcium, and almost all the vitamins of the B-group, with a prebiotic effect; and aleurone cells were the part of the bran most prone to degradation (37, 38).

The antioxidant activity determined in JE (87.23%) and CR (90.36%) (Table 1) were about four times higher than the antioxidant activity of wheat flour, also determined by the DPPH method (18.76 and 22.97%) (39). BENTO, Manoel S. S. Júnior (40) found similar antioxidant activity for JE flour (89 %) when evaluating the viability of this flour for use in the food industry. Moreover, Jatrophaelliptica extracts has demonstrated some biological activities related to the presence of tannins, saponins, phytates, nitrates, trypsin and amylase inhibitors (40, 42, 43). The presence of these compounds with emphasis on amylase inhibitors, and the high antioxidant activity may have negatively influenced production of enzymatic extracts.

Thus, CR and JE roots flour show high antioxidant activity as well as high concentration of phenolic compounds, nevertheless they cause an inhibition in CMCase and xylanase enzymes production. This indicates that the CR and JE flour would not be a suitable source of substrate for semisolid fermentation with the bacterium Streptomyces thermocerradoensis I3. As a result, the evaluation of pH influence was conducted only on the production of enzymatic extract using WB as substrate.

Determination of optimal pH

The influence of pH on CMCase production was evaluated and the lowest activity was found in pH 6.5 (1.95 ± 0.07 IU·mL^{-1}). In the neutral pH range (7.0), the activity was 8.07 ± 0.75 IU·mL^{-1} as showed in Figure 2A. These results corroborate to the data from Chellapandi and Jani (44), that showed maximum CMCase activity at pH 7.0 and 7.5 in extracts produced by two different Streptomyces species isolated from soil. These results are close to those found by Jang (45) that obtained an endoglucanase (CMCase) produced by Streptomyces T3-1 with optimum activity at pH 7.0. The work of Brito-Cunha, Gama (14) revealed the activity of CMCase, also using Streptomyces I3, in the pH range between 3.5 and 7.5. Moreover, Bispo, Andrade (46) used Streptomyces diastaticus PA-01 in submerged fermentation, demonstrating that 75% of endoglucanase activity is maintained over a wide pH range (2.0 to 8.0) with optimal activity occurring at three different pH values, varying according to the raw material used.

About the influence of pH on the activity of xylanases, a higher activity at neutral pH (4.03 ± 0.20 IU.mL^{-1}) was observed; while at pH 6.5 activity was 1.87 ± 0.10 IU·mL^{-1} and the lowest activity was observed at pH 7.5 (0.37 ± 0.07 IU·mL^{-1}), as showing in Figure 2B.

Chakdar, Kumar (47) reported that Streptomyces xylanases have optimal pH values in the range of 5.0 to 7.0. Brito Cunha, Gama (48) determined that the optimum pH value for purified r-XynS27 was found in 6.0, but the enzyme maintained 60% of its initial activities at pH 4.5 and 8.5. A first β-1,3-d-xylosidase (rSWU43A) isolated from a gram-positive bacterium, Streptomyces sp. SWU10, showed its optimal activity at pH 6.5 and the enzymatic activity remained above 75% of initial levels after incubation in a pH range of 3.1–8.9(49).

Enzymatic hydrolysis of Jatrhofa elipticaandCochlospermum regiumflour

To evaluate the efficacy of the enzymatic extract produced from the wheat bran substrate, enzymatic hydrolysis of the CR and JE flour was done. The solutions containing the CR, JE and the enzymatic extract produced from the bacterium Streptomyces thermocerradoensis I3 in semisolid wheat bran cultivation showed an increase, in both reducing sugar content and protein content (Figure 3).

In the first four hours of hydrolysis there were increases of 7 and 2.5 times in the contents of reducing sugars and soluble proteins, respectively. Then, there was a slower increase, which indicates that the enzymes acted on the molecules of the amorphous part of the cell wall, shortening them in smaller sugars, with reducing end (free carbonyl).

The increase in sugar content was due to the breakdown of polysaccharides into reducing sugars (50). Thus, the enzymes present in the extract were able to degrade compounds present in the substrate. However, the increase in the reducing sugar content was not enough for the subsequent application of this hydrolyzed to the production of alcoholic fermentation (preliminary tested), for example.

The concentration of fermentable sugars produced may have been influenced by the pH of the solution, since the enzymatic hydrolysis can be reduced or increased depending on some parameters, such as pH (PEREIRA et al, 2017a). At this research stage, only the temperature of the enzyme extract used was controlled (50° C). The

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increase in soluble protein content may have resulted from the release of polysaccharide-associated protein complexes and phenolic compounds, which were released form hydrolysis (52).

Enzymatic hydrolysis also promoted the increase in about 30% of the phenolic compounds in the flour with 48 h of exposure to the enzymatic extract and total soluble sugars (Figure 4).Phenolic compounds are secondary plant metabolites that may be complexed with cell wall components, food proteins, polysaccharides, fiber constituents and lipids. When complexed with these macromolecules, they are usually not extracted with the solvents used in the preparation of the extracts for the quantification of the phenolic compounds, nor are they quantified in the quantitative analysis of phenolics, since they might be retained in the matrix of food, inaccessible to the solvents due to different interactions with the plant matrix (53-55).

Polyphenols are responsible for a diversity of biological activities, including antioxidant and anti-inflammatory effects, prevention of neurological, cardiovascular and chronic intestinal diseases, diabetes and Alzheimer's disease and improvement of memory and cognitive function (56, 57). Thus, it can be inferred that the increase of the phenolic compounds in the CR flour that underwent the addition of the enzymatic extract was due to the hydrolysis of polysaccharides and consequent release of the phenolic compounds that were complexed to them. This increase can improve the bio- accessibility and bioavailability of these compounds. Anson, Selinheimo (58) and Mateo Anson, Aura (59) demonstrated that fermentation with hemicellulase (xylanase), β-glucanase, α-amylase and ferulic acid esterase in wheat bran increased phenolic acids bioavailability and the production of 3-phenylpropionic acid, the final product of the ferulic acid colonic metabolism.

IV. CONCLUSION

Wheat bran proved to be the best substrate to produce cellulases and xylanases using the bacterium Streptomyces thermocerradoensis I3, since the other substrates (Jatrhofa elipticaandCochlospermum regiumflour) did not supplied enzymatic extracts with satisfactory activity, nor presented activity for the two enzymes studied (cellulases and xylanases). The optimum pH to produce cellulases and xylanases using wheat bran as substrate, in semisolid fermentation with bacterium Streptomyces thermocerradoensis I3, was 7.5 and 7.0, for CMCase and xylanase, respectively. Moreover, the results suggest that the enzymatic assisted processing is promising in the production of flour enriched with polyphenols, contributing to a greater functionality with good nutritional and antioxidant properties.

SOME OF THE ADVANAGES FROM THE ABOVE RESULTS

a) Enzymatic extracts obtained by Streptomyces thermocerradoensis I3 show cellulase and xylanase activity.
b) Changing the pH of the culture medium allowed to favor higher production of cellulase or xylanase depending on the interest.
c) Cochlospermum regium and Jatrhopha elliptica flours were not found to be viable culture media when compared to wheat bran.
d) Enzymatic treatment with the extract obtained by Streptomyces thermocerradoensis I3 proved to be effective for the nutritional enrichment ofCochlospermum regium and Jatrhopha elliptica flours. Further studies are needed to prove the use of enzymatic extract to enrich other flours or other foods.

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Table 1. Phenolic compounds detected in different extracts of roots from Jatropha elliptica and Cochlospermum regium (wet basy)

	Mean	Standard deviation	Coefficient of variation (%)
Jatropha elliptica flour			
Antioxidant activity[1]	87.23	0.58	0.66
Phenolic compounds[2]	11.47	0.87	7.58
Cochlospermum regium flour			
Antioxidant activity[1]	90.36	0.94	1.04
Phenolic compounds[2]	8.32	0.45	5.40

[1] % of discoloration of DPPH; [2]mg eq gallic acid (g dry matter) $^{-1}$ on methanolic extract

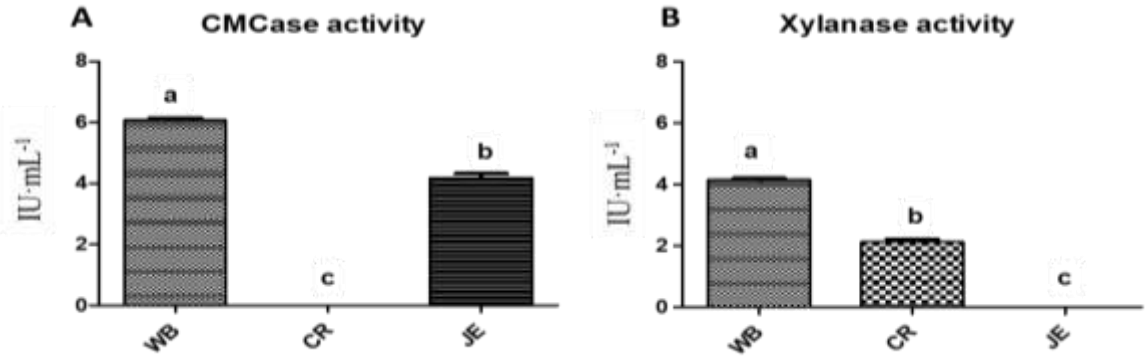

Figure 1. Enzymatic activities of CMCase (A) and xylanases (B) obtained from Streptomyces thermocerradoensis I3 using wheat bran (WB), Cochlospermum regium (CR) and Jatrhofa elliptica (JE) substrates after five days incubation, at 37°C.

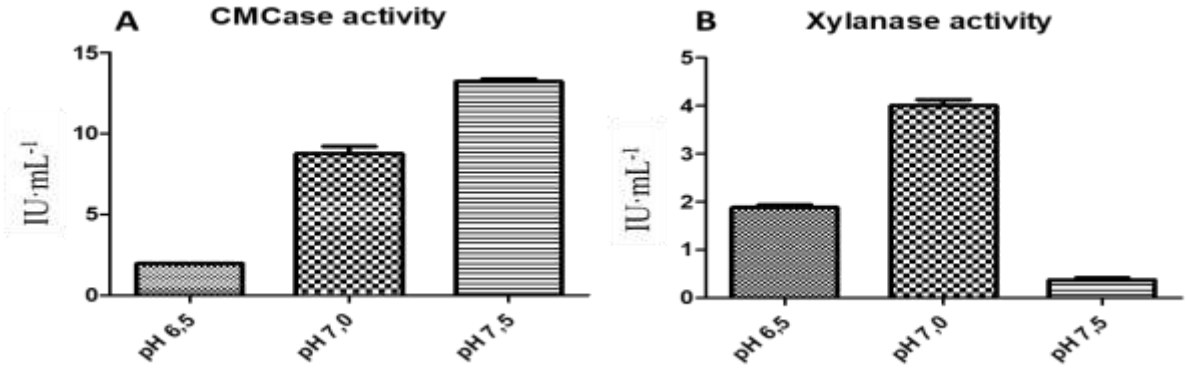

Figure 2. A: Enzymatic activity for Endoglucanase (CMCase) and B: Enzymatic activity of xylanase, obtained by semisolid fermentation from wheat bran substrate at three different pH values,after five days incubation, at 37°C.

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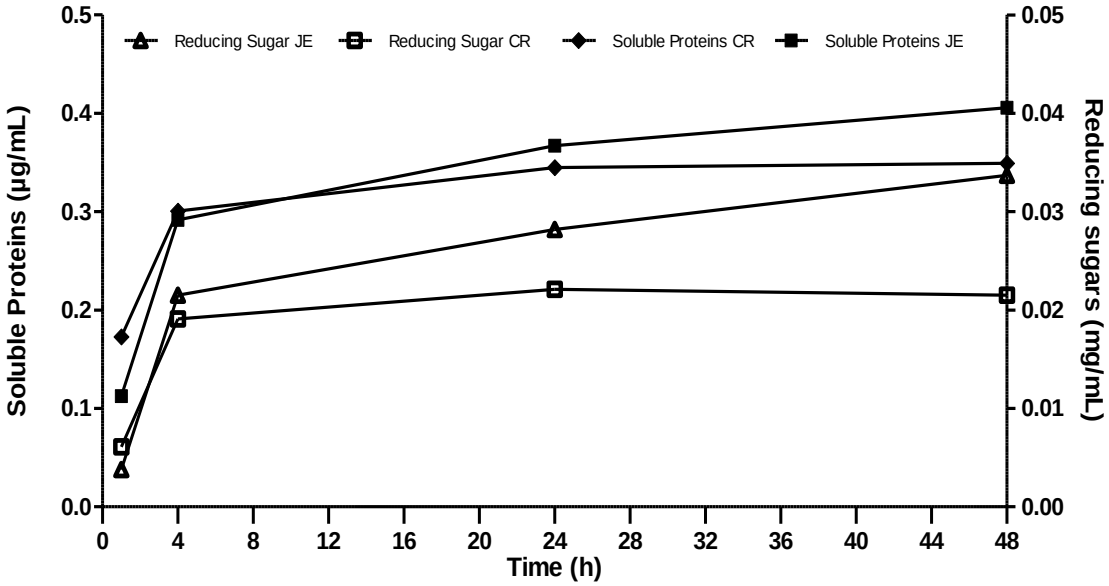

Figure 3. Reducing sugars and level of soluble proteins at times 0, 4, 24 and 48 hours incubation at 50°C in the Cochlospermum regium (CR) Jatrhofa eliptica (JE) flour. As a control, flour solutions were used without the addition of the enzyme extract.

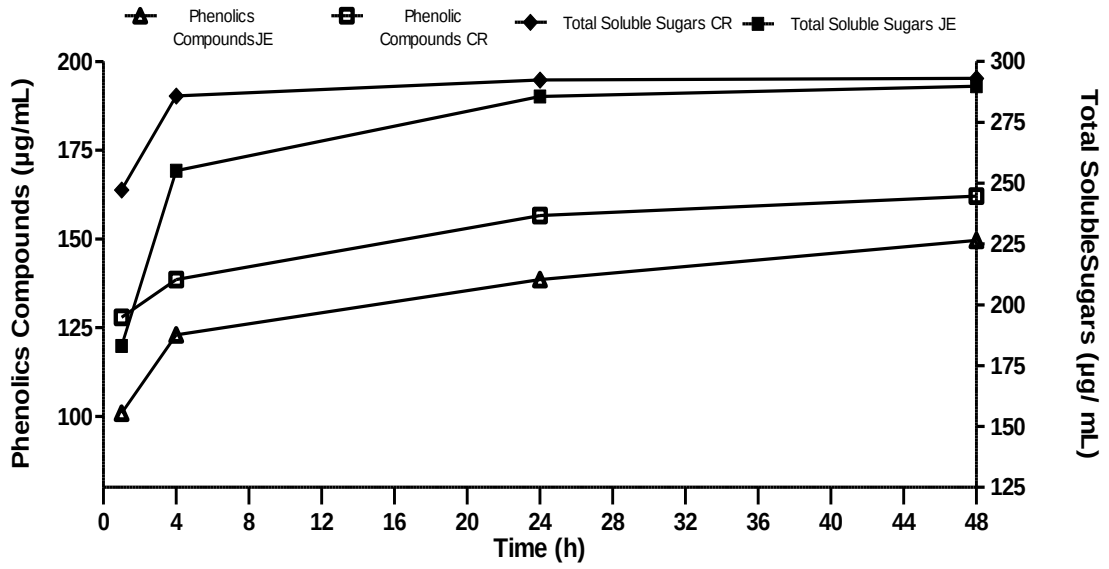

Figure 4. Phenolic compounds and level of total soluble sugars at times 0, 4, 24 and 48 hours incubationa at 50°C in the Cochlospermum regium (CR) Jatrhofa eliptica (JE) flour. As a control, flour solutions were used without the addition of the en