# BIOCONVERSION OF LIGNOCELLULOSIC MATERIALS WITH THE CONTRIBUTION OF A FERULOYL ESTERASE HYDROLASE FROM *Alternaria tenuissima* TO RELEASE CARBOHYDRATES

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## SUMMARY

Feruloyl esterase catalyze the hydrolysis of a wide variety of glycosidic linkages between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Thereby, they play a role in the initial degradation of lignocelluloses, which may be advantageous for the efficient degradation of the plant cell-wall complex that contains both diverse sugar residues and esterified structures. The contribution of the enzyme *Alt*FAE was purified from *Alternaria tenuissima* to the conversion of lignocellulosic materials without any chemical pretreatment to release the carbohydrates, was studied. It was determined the activity of feruloyl esterase (*Alt*FAE) production by *A. tenuissima* in liquid cultures with potential inducing agents sawdust, rice straw, tomato, potato, soybean, and corn starch. At the applied concentrations of soybean (2%), the highest levels ~ 689 U $L^{-1}$ of enzyme activities were obtained. The disintegrating effect of enzymatic lignocellulose treatment can be significantly improved by using different kinds of hydrolytic and oxidizing enzymes. Synergistic conversion of cane bagasse also resulted in a release of 18.4 mg $g^{-1}$ total carbohydrates (galactose, glucose, mannose, and xylose) per gram substrate using an "enzyme cocktail" [Cell/Xyl 50 U $gds^{-1}$, *Alt*FAE 10 U $gds^{-1}$, and Lac 15 U $gds^{-1}$] after incubating for 48 hrs at 45°C. The information presented in this paper is helpful to better understand the bioconversion of lignocellulosic materials with the contribution of a feruloyl esterase hydrolase from *A. tenuissima* to release carbohydrates.

**Keywords*:* Carbohydrate esterase, hydrolytic enzymes, lignocellulose bioconversion, oxidative enzymes.**

## 1. INTRODUCTION

Agricultural crops in Asia are primarily based on wet rice cultivation, the annual production of which leaves roughly 600 to 800 million tons of rice straw (lignocellulose) accounting for 90% of the worldwide amount (Abraham, *et al*., 2016). Despite the wide potential of rice straw, the challenging goal in converting this lignocellulosic biomass is to produce value-added chemicals at high selectivity and commercial effciency. Lignocellulose generally contains complex cell-wall polymers such as cellulose (32–39 wt%), hemicellulose (20–36 wt%), and lignin (14 wt%-22 wt%), along with silica and other minor components (10-17wt%) (Goodman, *et al*., 2020), which provide rigidity and mechanical stability and protecting the cell from microbial and enzymatic attack. Thus, this is the raw material for biotechnological and industrial applications, this vast resource becomes more and more economically important, not least against the background of intensified biomass utilization in the sense of the biorefinery concept and the idea of sustainable development (Aya and Paes, 2019). The lignocellulose outcomes from the plant synthesis of complex cell-wall polymers provide rigidity and mechanical stability and protect the plants from microbial attack. Since the process requires less energy in mild conditions, enzymatic hydrolysis is becoming a suitable pathway in biomass hydrolysis. Moreover, because of the rigidity and mechanical stability of lignocellulose, critical for successful use of this biomass is the development of enzyme cocktails that will break the plant cell wall down into usable fractions (Vasic *et al*., 2021).

In addition to the well-described oxidative enzymes (e.g., laccase, lignin peroxidase, manganese peroxidase) involved in lignocellulose degradation, several hydrolytic enzymes belonging to carbohydrate esterases are needed for complete conversion (Baldrian and Valaskova, 2008). Feruloyl esterase (FAE;

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EC 3.1.1.73) catalyzes the hydrolysis of a wide variety of glycosidic linkages between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety and thereby plays a role in the initial degradation of lignocelluloses. FAEs can be divided into 4 types (A-D) based on their specificity for the substrate aromatic moiety, their for the linkage to the primary sugar and their to release carbonhydrates (Crepin and Faulds, 2004). Since the 1990s, more than 30 FAEs have been purified and characterized from a variety range of microorganisms, including bacteria and fungi. Many enzymes from the genera *Aspergillus, Clostridium* and *Penicillium* have been studied. The purified FAEs showed significant variations in the physical characteristics and optimum hydrolytic reaction conditions (Topakas *et al*., 2007). Fungal strain *A. tenuissima* is a saprophytic fungus and opportunistic plant pathogen. It has a cosmopolitan in distribution, and can colonize a wide range of plant hosts such as blueberries, tomatoes, grapevine, strawberries (Domsch *et al*., 1980) and several cereal grain species. The aim of the study was lignocellulose hydrolysis of a FAE combination with Cellulase AB (Cell/Xyl) and oxidase Lacase (Lac) for the efficient conversion of lignocellulosic materials to release carbohydrates without chemical pretreatment.

## 2. RESEARCH METHODOLOGY

### *Materials*

- Lignocellulosic sources such as sawdust, rice straw, tomato, potato, soybean, and corn starch were provided by supermarket (Vietnam). All the above materials for conversion need to be dried, grinded and screened (0.5 - 1.0 mm size).

**Figure 1. Sugar cane bagasse after grinding**

Hydrolytic enzymes were purified from different fungi in previous studies:

- Commercial enzymes were purified from *Trichoderma reesei* (Carboxymethyl cellulase and glucuronoxylanase; Cell/Xyl, 1000 - 1600 U $g^{-1}$, AB Enzyme, Darmstadt, Germany).

- Laccase (Lac, 120 U $mg^{-1}$) was provided by Megazyme.

- Enzyme feruloyl esterase (*Alt*FAE, 11.2 U $mg^{-1}$, total activity: 151.2 U) was purified from *A. tenuissima* (Chi *et al*., 2017).

### *Fungus and Growth Conditions*

The wood decay ascomycete *A. tenuissima* was obtained from the HaUI Institute of Technology, Hanoi University of Industry (HaUI). The stock culture was maintained at 4°C on malt extract agar (MA) plates containing 20 gram of malt extract per litter after fungal mycelia fully covered the agar surface. The pre-culture was prepared by transferring an agar plug (Ø 1 cm) from stock culture onto a new MA plate and then incubated at 23°C. The 2-week-old pre-cultures were readily served for the subsequent experiments.

### *Liquid Cultivation*

Firstly, a minimal medium containing the essentials for fungal growth: 0.5 g of $MgSO_4$, 1.5 g of $KH_2PO_4$, 2.0 g of yeast extracts per 1 liter, was prepared. Thereafter, 100 ml Erlenmeyer flasks containing 50 ml of the above minimal medium (pH 6.0) each were

supplemented with 2% (w/v) of potential feruloyl esterase stimulating substances with lignocellulosic sources: sawdust, rice straw, tomato, potato, soybean, and corn starch. Afterward, 5 ml of the suspension was transferred into a liquid medium for incubation at 23ºC on a rotary shaker (200 rpm). During incubation of 12 days, aliquots (1 ml) were taken from fungal liquid cultures after intervals of 3 days for the measurement of esterase activity.

***Enzyme Assays***

FAE activity was determined by following the hydrolysis of 1 mM methyl ferulate to ferulic acid in 100 mM 3-(morpholino)-propane sulfonic acid (MOPS) buffer at pH 6.0 and 37°C. The reaction was initiated by the addition of enzyme solution and terminated after 5 to 30 min by an equal volume of the stop solution containing 11.3% acetic acid-methanol (vol/vol) (Faulds *et al*., 1994). The reaction product (ferulic acid) was analyzed by HPLC (isocratic elution from 10 to 15% acetonitrile; flow rate, 1 mL/min) using a reversed phase C18 column (250 × 4.6 mm, 5 µm, Alliance series 2695 Waters, USA) with diode array detector (DAD, λ = 323 nm).

Laccase activity was measured by the oxidation of 2,2'-azino-bis(3-ethylbenzthiazonline-6-sulphonic acid) (ABTS) to the corresponding cation radical as previously described (Liers *et al*., 2007).

**Isolation and Purification of *Alt*FAE**

The purification of *Alt*FAE from *A. tenuissima* was performed using an FPLC ÄKTA system (GE, Freiburg, Germany). The enzymatic extract from *A. tenuissima* culture was applied to different anion-exchange and size-exclusion chromatography steps as described previously (Chi *et al*., 2017).

**Hydrolysis of raw material by an enzyme cocktail containing *Alt*FAE**

Enzymatic reactions were performed under conditions as the previous study (Chi *et al*., 2012) in which the optimum temperature and pH of *Alt*FAE were found to be 45°C, pH 7 and incubation time for the conversion of the lignocellulosic materials to be for 48 hours. The cane bagasse materials was ground to a fine powder (0.5 - 1.0 mm size), suspended in distilled water (1%; w/v), and then used as stock solution. The different conditions were set based on the specific properties of materials and the product analysis as follows: Hydrolysis of finely milled cane bagasse was investigated using *Alt*FAE and further hydrolases and oxidoreductases, which were commercially available (AB Enzymes) or previously purified, to improve the efficiency of the conversion process. The cane bagasse powder (0.5%; w/v) was incubated with enzymes from *A. tenuissima* (10 U *Alt*FAE; 50 U Cell/Xyl and 15 U Lac) for 48 h at 45°C. The reaction was carried out in 100 mM MOPS buffer (pH 7.0), and for comparison purposes, a control with heat-inactivated enzyme (95ºC for 30 min) was used. To assess the synergistic and single effects of each enzyme, the enzymatic conversion was performed with each enzyme alone and in combinations on the one hand with all hydrolases (*Alt*FAE and Cell/Xyl) and on the other hand, with all tested enzymes (*Alt*FAE, Cell/Xyl, and laccase). Controls containing heat-denatured enzymes (95ºC for 15 min) were used.

***Thin layer chromatography (TLC)***

The products of the conversion reaction are monosaccarit (reducing sugars), determined by Thin layer chromatography. The solvent system used was n-butanol: aceton: $H_2O$ (8:1:2). Reagents $H_2SO_4$ (5-10%) is used to determine the spots of sugar on TLC. The standard sugars used were D-glucose, D-galactose, D-xylose and D-mannose (Sigma - USA).

***Analytical methods for hydrolysis products***

The products such as glucose, xylose, manose and galactose formed by hydrolysis were determined by HPLC (LC-10AD, Shimadzu Co., Kyoto, Japan) equipped with a refractive index detector (RID-10A, Shimadzu Co.) and ICSep WA-1 Wine Analysis column (Transgenomic, NE, USA) operated at 40°C with 1.25 mM sulfuric acid as mobile phase at fow rate of 0.6 mL $min^{-1}$.

## 3. RESULTS AND DISCUSSION

### Maximum activity of feruloyl esterase (*Alt*FAE) on different lignocellulose substrates

Production of *Alt*FAE on different lignocellulosic substrates was tested for the ascomycetous strain from *A. tenuissima*. This fungus was grown in liquid cultures supplemented with different lignocellulosic substances consisting of ester bonds and serving as the sole carbon source. Carbon source from sawdust, rice straw, tomato, potato, soybean, and corn starch remarkably stimulated hydrolytic production compared with control, which did not contain any inducer and none of the esterase activities. During the liquid-state cultivation of natural lignocelluloses, Fungi produced feruloyl esterase to a certain extent (256 – 689 U $L^{-1}$). At the applied concentrations of soybean and corn starch (2%), the highest levels (~ 689 and 623 U $L^{-1}$, respectively) of enzyme activities were obtained (Fig. 2). The pH of the culture medium slightly decreased from an initial pH 7.0 to a weak acidic pH of 6.2. Both substances seem to be the most suitable natural inducers to improve enzyme production. At the same concentration, Rice straw and Potato also had an enhancing effect that turned out to be approximately half of the maximal attained activities of wheat straw substrates (~ 623 U $L^{-1}$ and 573 U $L^{-1}$, respectively). Other tested carbon sources such as sawdust and tomato did not effectively stimulate the respective enzyme production. Most of the investigated carbon sources increased biosynthetic yield feruloyl esterase of the fungal strain. It is explained that, in carbon sources, cellulose is synthesized by simple sugars linked together by glycosides. In general, the feruloyl esterase activity of *A. tenuissima* culture filtrate was equal to or lower than that reported for these fungi, but enzyme production in the present study could be significantly stimulated using inexpensive and ubiquitous raw carbon sources like rice straw, soybean or corn starch.

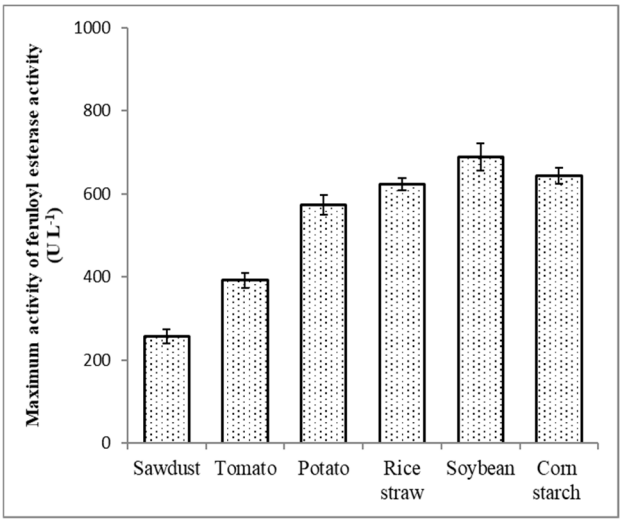


**Figure 2. Maximum activity of feruloyl esterase (*Alt*FAE) production by *A. tenuissima* in liquid cultures during 12-day incubation with potential inducing agents: sawdust, rice straw, tomato, potato, soybean, and corn starch. Aliquots were collected at time intervals of 3 days for enzyme activity measurement (error bars)**

### Release of carbohydrates from different substrates

The release of carbohydrates (monosaccarit) was evaluated on 5 different substrates: rice straw (M1), cane bagasse (M2), waste coffee grounds (M3), sawdust (M4) and algae (M5) by "enzyme cocktail" (Cell/Xyl = 50 U/gds, *Alt*FAE = 10 U/gds và Lac = 15 U/gds). The reaction was incubated for 48 hours at 45ºC. The hydrolysis products were determined by

thin layer chromatography (TLC). The results from Fig. 3 showed that samples M3, M4 and M5 did not appear any spot of monosaccarit. Meanwhile, both samples rice straw and cane bagasse, reducing sugars appeared very clearly. The experiment using cane bagasse as a substrate, on TLC showed dark spot with $R_f$ of 0.34 equal to glucose standards, other spots with $R_f$ is 0.28; 0.32; 0.48 equal to galactose, mannose, xylose standards, respectively. Therefore, cane bagasse was selected for further study.

**Figure 3. Carbohydrates appear on thin layer chromatography (TLC)**
***Glucose [$R_f$ (Glu) = 0.34], galactose [$R_f$ (Gla) = 0.28], xylose [$R_f$ (Xyl) = 0.48] and mannose [$R_f$ (Man) = 0.32]***

**Release of carbohydrates from cane bagasse**

A similar synergistic effect was observed for sugars release from cane bagasse meal by enzymatic treatment with single hydrolases or oxidases and in a combination of these enzymes. Table 1 showed the enzymatically released amounts of the four most abundant sugars in the cellulose and hemicellulose moieties of cane bagasse material after correction with the corresponding controls. The incremental amounts of 4.29, 6.12 and 6.71 mg $g^{-1}$ for glucose were be measured after treatment with AB enzyme, *Alt*FAE plus AB enzyme and all enzymes together (AB enzyme, *Alt*FAE and Laccase), respectively. Such a synergistic effect was also observed for galactose (0.5; 2.69; and 3.27 mg $g^{-1}$); Mannose (2.83; 3.94; and 3.96 mg $g^{-1}$), and Xylose (3.4; 3.63; and 3.87 mg $g^{-1}$).

**Table 1. Release of sugars from cane bagasse by enzymatic treatment**

| Reaction | Designation | Release of carbohydrates (mg $g^{-1}$) | | | |
|---|---|---|---|---|---|
| | | Glucose | Galactose | Mannose | Xylose |
| Hydrolase | *Alt*FAE | 1.92 | 0.94 | 0.63 | 1.95 |
| AB Enzymes | Cell/Xyl | 4.29 | 1.34 | 2.83 | 3.40 |
| Laccase | Lac | 0.12 | 0.08 | 0.07 | 0.13 |
| *Alt*FAE & AB Enzymes | *Alt*FAE & Cell/Xyl | 6.12 | 2.69 | 3.94 | 3.63 |
| *Alt*FAE; AB Enzymes & Laccase | *Alt*FAE; Cell/Xyl & Lac | 6.71 | 3.27 | 3.96 | 3.87 |

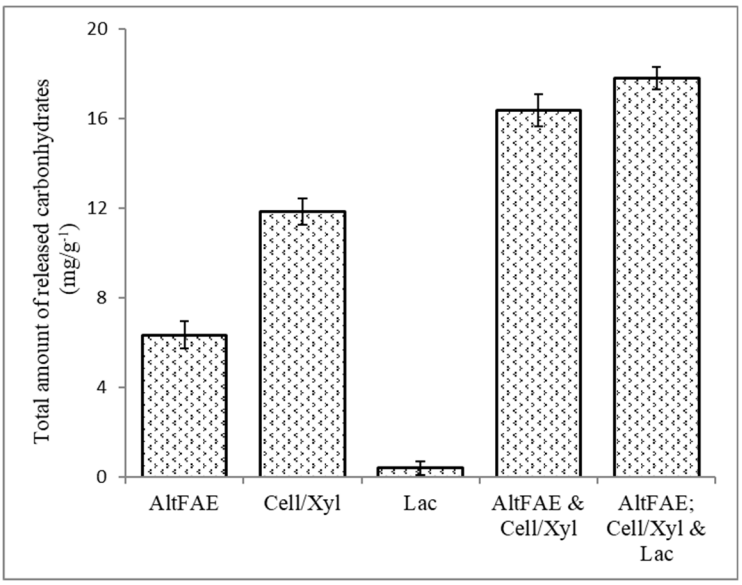


**Figure 4. Total amount of the released carbohydrates from milled cane bagasse after treatment with different enzymes (*Alt*FAE; Lac; and Cell/Xyl) as single applications, and in two enzyme combinations (*Alt*FAE and Cell/Xyl; *Alt*FAE, Cell/Xyl and Lac). The values are corrected by the sugar amounts in the corresponding controls containing heat-denatured enzymes**

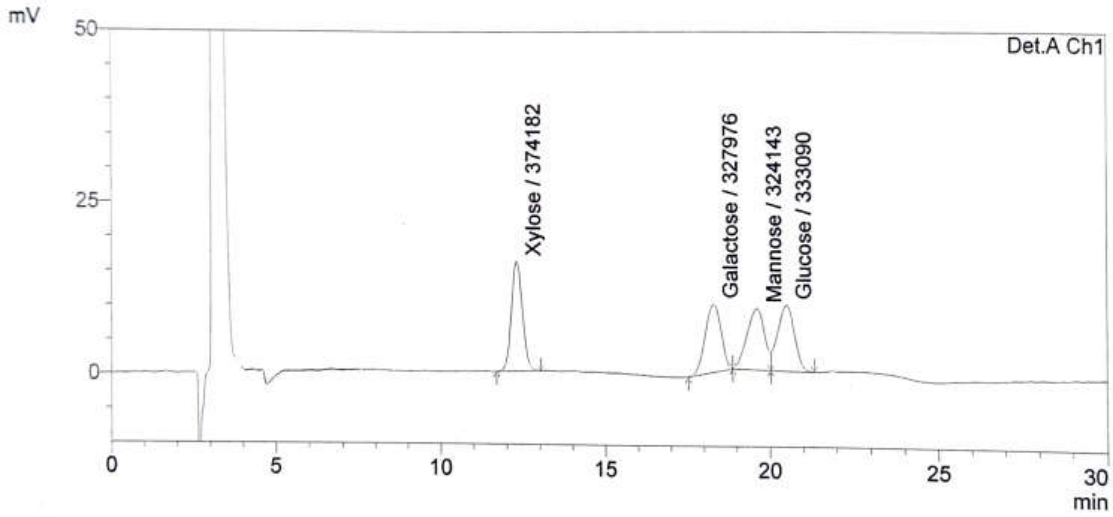


**Figure 5. HPLC elution profiles of monosaccarit released from cane bagasse after 48 hours incubation with a combination of enzymes: *Alt*FAE (10 U); Cell/Xyl (50 U); and Lac (15 U)**

A comparison of the sum of all four detected carbohydrates released by each enzymatic reaction system was shown in Fig. 4. The effect caused by *Alt*FAE (total amount of released carbohydrates: ~ 8 mg $g^{-1}$) is half of that observed for the cellulase/xylanase cocktail (Cell/Xyl; 12 mg $g^{-1}$). It was no surprise that the oxidase Lac alone did not affect sugar liberation from cane bagasse meal. Notably, the overall carbohydrate release increased up to 17 mg $g^{-1}$ using a combination of *Alt*FAE and the crude cellulase/xylanase preparation. The addition of Lac to this enzyme cocktail containing only hydrolases further increased the effect up to 18.4 mg $g^{-1}$. Moreover, the synergetic action of the tested enzymes was proved by the release of C-5 and C-6 carbohydrates (glucose, xylose, galactose and mannose) from cane bagasse powder, which was enhanced by using in combination with all hydrolytic enzymes combination of all hydrolytic enzymes (Cell/Xyl and *Alt*FAE) or additionally with Lac,

although Lac alone did not affect the liberation of sugar from cane bagasse. Vancov and McIntosh (2012) demonstrated the increase in sugar release from ~ 135 to 190 mg $g^{-1}$ by doubling the reaction temperature from 60 to 121°C.

Enzyme cocktail was used to convert the raw cane bagasse material, which contained about 45.8 wt% cellulose 23.2 wt% hemicellulose, and 24.3 wt% lignin (Pandey *et al*., 2000). After 48 hrs incubation, the release of carbohydrates (glucose, xylose, galactose and mannose) from the bagasse was detected by HPLC, as shown in Fig. 5. The efficiency of enzymatic hydrolysis of lignocellulosic material depends on the enzyme amount or substrate used. Therefore, the reaction parameters for the cane bagasse conversion by *Alt*FAE with and without accessory enzymes can be further optimized to improve the release of economically important metabolites.

## 4. CONCLUSIONS

Our study was determined the activity of feruloyl esterase (*Alt*FAE) production by *Alt. tenuissima* in liquid cultures with potential inducing agents: sawdust, rice straw, tomato, potato, soybean, and corn starch. At the applied concentrations of soybean (2%), the highest levels ~ 689 U $L^{-1}$ of enzyme activities were obtained.

Carbohydrates (monosaccarit or reducing sugars) released from cane bagasse was the highest among five investigated substrates, including: rice straw, cane bagasse, waste coffee grounds, sawdust and algae by "enzyme cocktail" (Cell/Xyl: 50 U/gds, *Alt*FAE: 10 U/gds and Lac: 15 U/gds). The overall carbohydrate release increased up to 18.4 mg $g^{-1}$ using a combination of *Alt*FAE with Cell/xyl and Lac.

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# CHUYỂN HÓA SINH HỌC VẬT LIỆU LIGNOCELLULOSIC THÀNH CARBOHYDRATES VỚI SỰ THAM GIA CỦA ENZYME THỦY PHÂN FERULOYL ESTERASE TỪ *Alternaria tenuissima*

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## TÓM TẮT

Feruloyl esterase xúc tác quá trình thủy phân nhiều loại liên kết glycosidic giữa hai hoặc nhiều cacbohydrat hoặc giữa một gốc cacbohydrat và một gốc không phải cacbohydrat. Vì thế, chúng đóng một vai trò quan trọng trong giai đoạn đầu của quá trình thủy phân lignocelluloses, điều này có thể có lợi cho quá trình chuyển hóa hiệu quả thành tế bào thực vật chứa các liên kết glucosidic và este hóa. Sự đóng góp của enzyme *Alt*FAE được tinh sạch từ nấm *Alternaria tenuissima* trong việc chuyển đổi các vật liệu lignocellulosic mà không cần tiền xử lý hóa học để giải phóng carbohydrate đã được nghiên cứu. Hoạt độ feruloyl esterase (*Alt*FAE) bởi *A. tenuissima* trong môi trường nuôi cấy lỏng với các chất gây cảm ứng tiềm năng là mùn cưa, rơm rạ, cà chua, khoai tây, đậu tương và tinh bột ngô. Khi sử dụng cơ chất đậu tương (2%) bổ sung vào môi trường nuôi cấy, hoạt độ enzyme *Alt*FAE thu được cao nhất ~ 689 U $L^{-1}$. Hiệu quả chuyển hóa lignocellulose bằng enzym có thể được cải thiện đáng kể bằng cách sử dụng các thủy phân và oxi hóa khác nhau có tác dụng hiệp đồng. Sự chuyển đổi bã mía đã giải phóng 18,4 mg $g^{-1}$ carbohydrate (galactose, glucose, mannose và xylose) trên mỗi gam cơ chất bằng cách sử dụng "enzyme cocktail" [Cell/Xyl 50 $gds^{-1}$, AltFAE 10 U $gds^{-1}$ và Lac 15 U $gds^{-1}$] sau khi ủ 48 giờ ở 45$^{o}$C. Thông tin trong bài báo này rất hữu ích nhằm hiểu rõ sự chuyển đổi sinh học vật liệu lignocellulosic thành carbohydrates với sự tham gia của enzyme thủy phân feruloyl esterase từ nấm *A. tenuissima.*

**Keywords: Carbohydrate esterase, hydrolytic enzymes, lignocellulose bioconversion, oxidative enzymes.**

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