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Enzyme Production of *Trichoderma reesei* Rut C-30 on Various Lignocellulosic Substrates

Chul Seung Shin, Joon Pyo Lee, Jin Suk Lee, and Soon Chul Park*

Biomass Research Team, New and Renewable Energy Department, Korea Institute of Energy Research, 71-2 Jangdong Yusonggu, Taejon, Korea, E-mail: bmscpark@kier.re.kr

Abstract

Economical production of cellulase enzyme is key for feasible bioethanol production from lignocellulosics using an enzyme-based process. On-site cellulase production can be more feasible with the process of separate hydrolysis and fermentation (SHF) than with simultaneous saccharification and fermentation, since the cost of enzyme is more important and a variety of substrates are available for the SHF process. Cellulase production using various biomass substrates available for SHF, including paper sludge, pretreated wood (steam exploded), and their hydrolysis residues, was investigated in shake flasks and a fermenter for their productivities and titers. Among the newspaper sludge, office paper sludge, and steam-exploded woods treated in various ways, the steam-exploded wood showed the best properties for substrate in cellulase production. The best titer of 4.29 IU/mL was obtained using exploded wood of 2% (w/v) slurry in the shake flask, and the titer with the same substrate was duplicated to about 4.30 IU/mL in a 3.7-L fermenter. Also, the yield of enzyme reached 215 IU/g of substrate or 363 IU/g of cellulose. Despite various pretreatment attempts, newspaper and office paper substrate was inferior to the exploded-wood substrate for cellulase production. However, hydrolysis residues of papers showed quite promising results. The hydrolysis residue of office paper produced 2.48 IU/mL of cellulase in 7 d. Hence, the utilization of hydrolysis residues for cellulase production will be further investigated in the future.

Index Entries: Cellulase production; lignocellulosic substrates; waste papers; steam-exploded wood.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Economic evaluation of a future full-scale plant for production of ethanol from various lignocellulosic materials, such as softwood, hardwood, waste paper, and wheat straw, is frequently based on enzymatic hydrolysis instead of conventional acid hydrolysis because of the higher ethanol yield and lower by-product formation *(1)*. Moreover, modeling and sensitivity analysis of ethanol production process economics has shown the outstanding cost of enzyme production *(2)* and enzyme production facilities *(3)*.

Hence, economical enzyme production from proper substrates is key to developing enzyme-based bioethanol production from lignocellulosic biomass. Even though the commercial cellulase complex is normally utilized in the test run of a pilot plant for ethanol production, until now, the utilization of lignocellulosic substrate for the on-site production of cellulase enzyme and ethanol could not provide a streamlined and economic ethanol production process with reduced production cost *(4)*. Cellulase production from pure cellulose, or from soluble sugars such as lactose, cellobiose, and sophorose, was studied thoroughly in submerged culture by a number of investigators. However, the production of the enzyme with those high-value substrates was not economically feasible for the large-scale ethanol production process. Hence, wheat straw *(5)*, bagasse *(6)*, aspen wood *(7)*, willow *(8)*, yellow poplar *(4)*, and waste newspaper *(9–11)* treated by physicochemical methods such as alkali treatment, steam explosion, pulverizing, or partial hydrolyzation were utilized as substrates to produce effectively the cellulase complex. Also, sulfite liquor *(12)* and corn steep liquor *(13)* were added to those substrates to induce and improve the productivity of the enzyme.

The aims of this study were to determine not only the substrates available for the economical production of cellulase complex but also the methods of the pretreatment of each substrate including waste newspaper, office paper, and oak wood. The paper wastes were treated by ammonia with hydrogen peroxide catalysts. On the other hand, the oak wood chip was steam exploded after dilute acid percolation or without percolation. In addition, the residue of the hydrolysis was examined as substrate for the production of the enzyme.

Materials and Methods

Substrates and Their Preparation

Waste Newspaper and Office Paper

Waste newspaper pulverized with a Wiley mill (Thomas Scientific model 3383, –40 mesh) and ball mill, disintegrated by a pulper (helical blade type, Korea), was utilized for the production of cellulase. The newspaper disintegrated by the pulper was further treated with ammonium hydroxide (30% [w/v] NH_3) with and without hydrogen peroxide (H_2O_2)

as catalyst: newspaper ammonia treated at 170°C for 14 h, newspaper H_2O_2 (0.3% w/v) treated for 1 h and subsequently ammonia treated for 14 h, and newspaper H_2O_2 (1.0% w/v) treated for 1 h and subsequently ammonia treated for 1 h. Office paper disintegrated by the pulper was used as substrate without further treatment. Treated substrate samples were washed sufficiently with deionized water, dried, and subsequently pulverized by a Wiley mill (–40 mesh) before utilization.

Oak Wood Chips

Two types of steam-exploded wood chips were utilized: oak wood chips impregnated in 0.2% sulfuric acid for 14 h and subsequently steam cooked at 215°C for 3 min and exploded; and oak wood chips impregnated in 0.05% (w/v) sulfuric acid for 14 h and subsequently percolated with 0.05% sulfuric acid (500 mL/min flow rate, 2.2-kg wood chips in 12 L) at 170°C for 60 min and exploded (at 215°C, for 30 s). The exploded wood chips were washed sufficiently, dried, and pulverized below 40 mesh to exclude the effect of the size of the solid substrates.

Hydrolysis Residue

The residues of enzymatic hydrolysis of disintegrated newspaper, office paper, and steam-exploded wood chips were also utilized for the production of cellulase. The residues were obtained by the following procedure. Disintegrated newspaper, office paper, or steam-exploded wood chips (215°C for 3 min after 0.2% [w/v] H_2SO_4 impregnation overnight) were hydrolyzed with the addition of Cellusoft (20 IU/g of substrate) and Novozym 188 (30 IU/g of substrate) at 50°C for 96 h with an initial pH of 4.8. The residues of the hydrolysis were washed, centrifuged, dried (at ambient temperature), and pulverized (–40 mesh), and were then utilized for cellulase production testing.

Strains and Cultivation

Trichoderma reesei Rut C-30 (ATCC 56765) was used for cellulase production. Seed cultures were prepared with a shaking incubator culture for 2 d at 30°C at 150 rpm in a 250-, 500-, or 1000-mL Erlenmeyer flask containing Vogel media of lactose (1% w/v). The seed cultures were inoculated with 10^6 spores/mL cultured on solid media (potato dextrose agar) for 7 d at 30°C.

Test cultivations in 250-mL Erlenmeyer flasks were made with 100 mL of Vogel media (initial pH 5.0) containing specified concentrations (1–3% w/v) of various substrates. Prepared flasks were inoculated with 5 mL of seed culture and then placed in a shaking incubator at 30°C at 150 rpm. A cellulase production experiment was conducted at a working volume of 2 L in a 3.7-L fermenter (Bioengineering, Switzerland) filled with the Vogel media of 2% (w/v) carbon sources and inoculated at 5% (v/v) with seed culture. The fermenter was operated at 30°C and 200 rpm with a 0.3-vvm airflow rate without pH control at an initial pH of 5.0.

Table 1
Composition of Substrates

Sample	Composition (% w/w solid)			
	Cellulose	Hemicellulose	Lignin	Misc.[a]
Waste newspaper disintegrated	53	17.0	25.0	4.7
Waste newspaper pretreated, in 30% NH_4OH, 14 h at 170°C	64	12.0	18.5	5.5
Pretreated in 1.0% H_2O_2, 1 h and 30% NH_4OH, 1 h at 170°C	65	8.3	19.8	6.9
Pretreated in 0.3% H_2O_2, 1 h and 30% NH_4OH, 14 h at 170°C	59	6.1	33.1	1.8
White office paper disintegrated	71	14.0	12.0	5.0
Steam-exploded oak wood chips at 215°C for 3 min[b]	59	7.0	29.6	4.4
H_2SO_4 (0.05%) percolation, 1 h at 170°C and exploded[b]	50	12.5	31.6	5.9

[a]Includes ash and resins and so forth.
[b]The composition of raw oak wood chips was as follows: cellulose, 49.3%; hemicellulose, 25.9%; Klasson lignin, 21.7%; miscellaneous, 3.1%.

Analysis

Cellulase complex (filter paper activity), CMCase, and β-glucosidase activities were measured by the IUPAC method *(14)*; that is, the activities were indicated in micromoles of glucose generated for 1 min using filter paper (Whatman no. 1), carboxymethyl cellulose (medium viscosity, Sigma), and cellobiose (Sigma) as standard substrate, respectively. The soluble protein, an indicator of both the cell mass and the enzyme released, was measured by the Bradford *(15)* method using the centrifuged precipitate of 2-mL culture samples extracted with 4 mL of acetone overnight at 4°C. The precipitates were dissolved in 0.05 *M* citrate buffer (pH 4.8) before protein assay.

Results and Discussion

Composition of Substrates

Table 1 presents the composition of substrates utilized in the cellulase production experiments and shows that pretreatment generally reduces hemicellulose (acid percolation, ammonia treatment) and lignin (ammonia treatment) contents of the substrates. It was expected that the changes of the composition and the structure of substrates would eventually enhance productivity of cellulase. Enzymatic hydrolysis residue of waste newspaper, white office paper, and steam-exploded wood were also tested for their performance as cellulase production substrates.

Flask Cultures

Waste newspaper and white office paper were subjected to various pretreatments such as pulverizing by ball mill or Wiley mill, simply disintegrating by a pulper at ambient temperature with water addition, and treating with ammonia with hydrogen peroxide catalysis. The cellulase activity and its productivity were measured for a flask culture of *T. reesei* Rut C-30 with those substrates. Figures 1 and 2 present the results of the experiment.

The cellulase titers showed their maximum after about 7 d of flask culture with all types of paper substrates. Ball mill and Wiley mill pulverizing did not made significant differences in cellulase productivity; however, the ball mill–treated papers showed slightly higher productivity at the early stage of cultivation than Wiley mill–treated papers. Waste newspaper and white office paper did not show much difference as a carbon source for cellulase production. The ammonia-treated waste newspaper with hydrogen peroxide showed an adverse effect for cellulase production; cell growth was inhibited (data not shown). Cellulase productivity was lower with ammonia-treated (Fig. 2) than with untreated samples (Fig. 1). Increased addition of hydrogen peroxide and longer treatment time did not show any significant improvement in cellulase productivity. The increased substrate concentration from 1 to 2% apparently inhibited cellulase production. In comparison with our data concerning wood substrates in the next section (Fig. 3), the ammonia-treated newspaper substrates were estimated to contain some inhibitory materials against cellulase production. In summary, the results with waste papers showed that the treatment enhancing cellulose content by removing hemicellulose or lignin in papers was not effective. On the contrary, it was believed that cellulase production was inhibited probably by by-products generated during the pretreatment.

Wood samples pretreated by steam explosion (215°C, 3 min) and percolation (170°C, 60 min, by 0.05% H_2SO_4) showed good characteristics as carbon sources for cellulase production (Fig. 3). The strain was well grown (data not shown) on steam-exploded oak wood substrate and produced 4.29 IU/mL of cellulase at the final stage of culture (10 d) with a 2% (w/v) carbon source concentration. The yield of enzyme reached 215 IU/g of substrate or 363 IU/g of cellulose with steam-exploded wood. Acid-percolated wood samples showed a slightly lower cellulase yield (163 IU/g of substrate) at a 2% substrate concentration. However, the cellulase yields with acid-percolated wood were much higher than those with waste paper substrates. And, in the case of wood samples, the cellulase titer was higher with a 2% concentration than with a 1% substrate concentration.

The use of hydrolysis residue of waste papers or exploded wood was considered to reduce the substrate cost of cellulase production (Fig. 4). From the data shown in Fig. 4, hydrolysis residues of newspaper and office paper produced almost equal or more cellulase than pulverized original papers (Fig. 1) when they were used as carbon sources for *T. reesei*. Similar

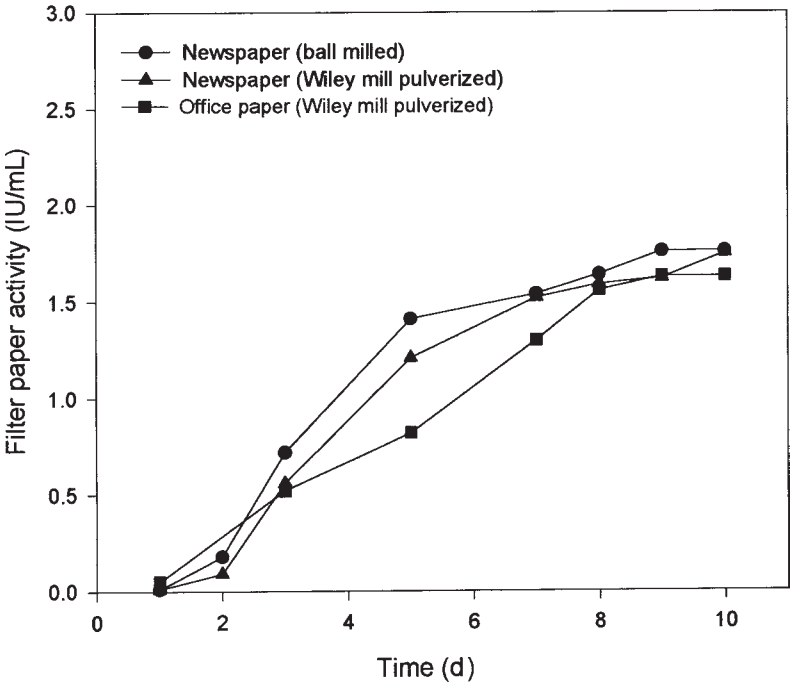

Fig. 1. Flask culture cellulase production of *T. reesei* Rut C-30 with pulverized 2% (w/v) newspaper and office paper sludges.

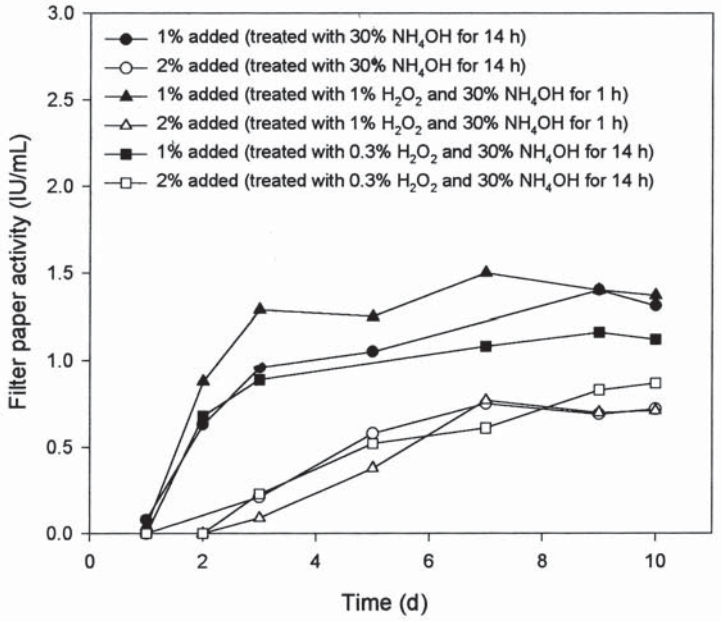

Fig. 2. Flask culture cellulase production of *T. reesei* Rut C-30 with newspaper and office paper pretreated by ammonium hydroxide with and without hydrogen peroxide catalysis at 1 or 2% (w/v) substrate concentration.

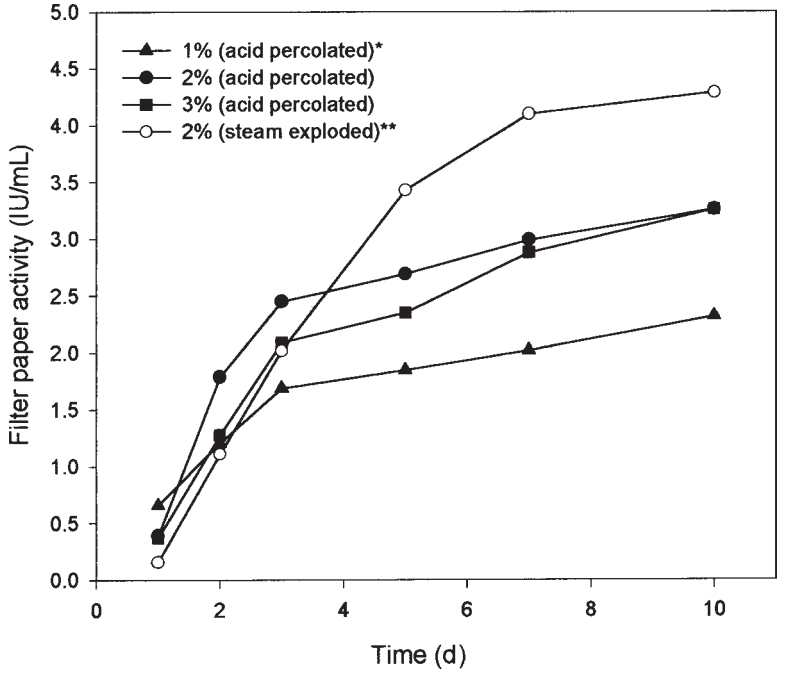

Fig. 3. Flask culture cellulase production of *T. reesei* Rut C-30 with steam-exploded and acid-percolated oak wood at various substrate concentrations. *, Acid percolated at 170°C, 1 h with 0.05% H_2SO_4 and exploded; **, steam exploded at 215°C for 3 min.

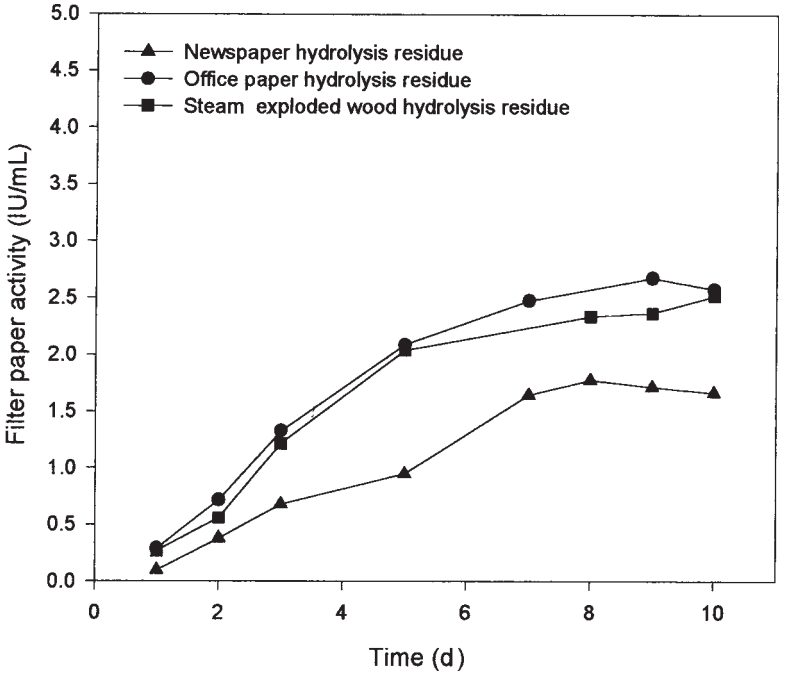

Fig. 4. Flask culture cellulase production of *T. reesei* Rut C-30 with hydrolysis residues.

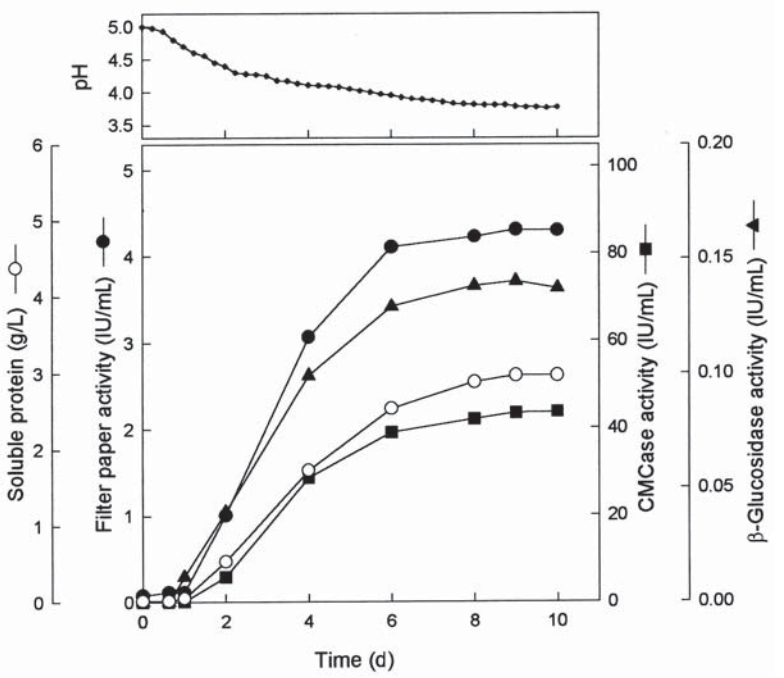

Fig. 5. Cellulase production with *T. reesei* Rut C-30 in fermenter using 2% (w/v) steam-exploded wood substrate.

results were published by Chen and Wayman *(9)*. They reported that partially hydrolyzed newspaper showed higher cellulase production than acid or alkali-treated newspapers when it was used as a carbon source. The enhanced production of cellulase with the hydrolysis residue of office paper was believed to be owing to the cellulose with low molecular weight generated during hydrolysis. On the other hand, the hydrolysis residue of exploded wood produced less cellulase than intact exploded wood. The reduced production of cellulase in this case was assumed to be the result of significantly reduced cellulose content in the hydrolysis residue of wood.

Fermentation with Exploded Oak Wood

Exploded wood, which showed high productivity in flask culture, was utilized for the production of cellulase in a fermenter. We experimented with batch fermentation in a 3.7-L fermenter with a 2.0-L working volume by adding 2% (w/v) exploded wood in Vogel media by seeding with 200 mL of preculture. Figure 5 presents the results of this fermentation. The production of cellulase was commenced within 24 h and was increased rapidly until d 6 to reach the maximum cellulase titer of 4.3 IU/mL. At this time, CMCase and β-glucosidase activity were measured to be 43.7 and 0.14 IU/mL, respectively. The balance of CMCase and β-glucosidase activity in the cellulase complex produced requires the addition of β-glucosidase for the maximum performance of cellulose degradation to glucose.

Acknowledgment

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References

1. Vallander, L. (1999), Newsletter for IEA Task 26, Biotechnology for the Conversion of Lignocellulosics to Ethanol, no. 4, March.
2. Desmarquest, J. P. and Requillart, V. (1989), Proceedings of the 5th E.C. Conference on Biomass for Energy and Industry, 9–13 Oct., Lisbonne, Portugal.
3. Nguyen, Q. A. and Saddler, J. N. (1991), *Bioresour. Technol.* **35,** 275–282.
4. Hayward, T. K., Hamilton, J., Templeton, D., Jennings, E., Ruth, M., Tholudur, A., Mcmillan, J. D., Tucker, M., and Mohagheghi, A. (1999), *Appl. Biochem. Biotechnol.* **77/79,** 293–309.
5. Chahal, P. S., Chahal, D. S., and Le, G. B. B. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 433–442.
6. Kawamori, M., Morikawa, Y., Ado, Y., and Takasawa, S. (1986), *Appl. Microbiol. Biotechnol.* **24,** 454–458.
7. Kan, A. W. and Lamb, K. A. (1984), *Biotechnol. Lett.* **6,** 663–666.
8. Szengyel, Z., Zacchi, G., and Reczey, K. (1997), *Appl. Biochem. Biotechnol.* **63/65,** 351–362.
9. Chen, S. and Wayman, M. (1991), *Process Biochem.* **26,** 93–100.
10. Doppelbauer, R., Esterbauer, H., Steiner, W., Lafferty, R. M., and Steinmuler, H. (1987), *Appl. Microbiol. Biotechnol.* **26,** 485–494.
11. Viesturs, U., Leite, M., Treimanis, A., Eremeeva, T., Apsite, A., Eismonte, M., and Jansens, P. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 349–360.
12. Qu, Y., Zhao, X., Gao, P., and Wang, Z. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 363–368.
13. Farid, M. A. and El-Shahed, K. Y. (1993), *Zentralbl. Mikrobiol.* **148,** 277–283.
14. Ghose, T. K. (1987), *Pure Appl. Chem.* **59,** 257–268.
15. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.