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Microbial Conversion of Woody Waste into Sugars and Feedstuff(I)

Optimal Delignification condition with alkaline peroxide for enzymatic hydrolysis of poplar wood*1

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微生物에 의한 木質資源의 糖化및 飼料化에 關한 硏究(I)

alkaline peroxide에 의한 현사시나무의 효소가수분해를 위한 탈리그닌화의 적정조건*1

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要 約

성(省) 에너지적 관점에서 목질자원의 식량자원화의 가능성을 검토하기 위하여 속성수종인 현사시나무를 공시수종으로하여 alkaline peroxide를 사용하여 탈리그닌화의 적정조건을 규명하였던 바, 25°C에서 100시간 동안 1% H_2O_2(pH 11.5)로 반응시킨것이 당화효율과 분해율이 가장 높았다. 이 조건하에서 생산된 당은 주로 glucose and xylose로 구성돼 있으며, 당화효율과 분해율 무처리재에 비해 각각 260%와 350%의 증가를 나타냈으며, 이같은 조건은 1% NaOH와 20% Peracetic acid로 전처리한 목분의 그것과 대비될만한 것이다.

Summary

Alakline peroxide pretreatment for the delignification of poplar wood was performed, sinceit is a simple and efficent method for enhancing the enzymatic digestibility of wood residues. Approximately one-half of their lignin and most of the hemicellulose present in poplar wood were removed when the wood sawdust was reacted at 25°C for 100 hrs in an alkaline solution (pH 11.5) of 1% peroxide. The rate of decomposition as well as the saccharification efficiency were enhanced up to 350% and 260% respectively in comparision with those of the controll. This enhancement is comparable with that pretreated with 1% sodium hydroxide and 20% peracetic aicd successively. The advantages of alkaline peroxide as delignifying agents against other chemicals were also discussed.

1. Introduction

The heterogenous decomposition of lignocelluloses is strongly influenced by their structural features, including crystallinity of cellulose, lignin content and the surface area of biomass. These structural and chemical barriers should be eliminated, to be able to utilize the lignocelluloses(e.g. wood sawdust)as a source for glucose and other sugars as well as a feedstuff for ruminant

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animals.[4,6] These have resulted in the development of various "pretreatments" to increase hydrolysis susceptibility before enzymatic hydrolysis. Most of the methods used include quite severe chemical or physical treatments which either remove the lignin or which drastically reduce the molecular order of the natural polymer [2,28]. While many pretreatments work well, they are not cost effective; they require high energy input and expensive chemicals as well as special facilities[16].

In this regard, among the various processes which have been proposed for altering or destroying lignin and thereby making the wood more digestible, we have examined the use of peroxide alkaline. This process presents various advantages ;1) it is exceedingly well suited for scaling down to comparatively small operations,2) neither high temperature nor pressure are involved and 3) the only chemicals necessary, peroxide and sodium hydroxide, can be chiefly purchased[1,12-14,18]. Thus, combined with free from envrionmental problems involving toxic wastes and the possibility of using the products for animal feed, make it attractive for local use with various types of biomass which is available in steady small supply, e. g. sawmill waste or rice straw etc.

A hybrid poplar (Populus alba x p.glandulosa)was used as lignocellulosic materials in the present work because it is not only easily available but widely planted in this country due to its fast growing character. We report the chemical components of hybrid poplar and optimal delignification condition with alkaline peroxide solution for the enzymatic hydrolysis and for the use of ruminant feedstuff.

2. MATERIALS AND METHODS

2.1 Lignocellulosic substrate

Poplar wood(Populus alba x P.glandulosa)was hammer milled and sieved. The fraction of saw dust, passing 20 mesh but retained by 40 mesh, was used as the standard substrate.

2.2 Analytical methods

Extractives and ash content of poplar wood was determined by the standard method of TAPPI. Cyclo hexane was used in the organic solvent extraction instead of benzene because of its carcinogenic effect[8]. The pH of poplar wood was determined by the method of Stamm[25]. The holocellulose content was determined by the method described by Wise et al[29] and that of Klason lignin by the method of Effland[5]. The content of acid soluble lignin in the filtrate of Klason lignin was determined with the hexaflluropropanol at 205nm[27]. Milled wood lignin from birch was used for the calibration. To estimate the nutritive value of poplar wood as feed stuff, chemical composition of this sawdust were also determined by the method described by Goering and van Soest[11] Monosaccharides were analyzed by the use of HPLC (Waters Associates Model 6000 A) with the solvent of acetonitrile : water(85 : 15) after the hydrolysis with triflur-oacetic acid(TFA)[10].

2.3 Pretreatment with alkaline peroxide

Delignification with alkaline peroxide was performed using 1%H_2O_2 solution[12]. With the addition of NaOH the pH of the reaction mixture was adjusted to 11.5. Five grams of saw dust were mixed with 250ml of aikaline peroxide solution. This mixture was reacted in different temperatures(25℃, 50℃, 75℃ and 120℃) for different lengths of time. After the reaction sample was filered through glass filter and the concentration of peroxide in the filtrate was determined with 0.1 N sodium thiosulfate. The pH-value during and after the reaction was checked by pH-meter. For the comparision of the pretreatment with other processes, poplar wood was also delginified by the method of Wise et al[29] and the method of Toyame and Ogawa[26].

2.4 Enzymatic hydrolysis

Substrate delignified under the different conditions were vacuum dried and 4 gr were weighed into a 200 ml flask, after which 80ml of 0.1M Citrate buffer(pH 5.0) and 20㎖ of the 1% cellulase enzyme solution(Cellulosin AP+Cellulase Onozuka R-10,1:1) were added to the flask to obtain a 4% substrate suspension.[7,21] The flask was placed in a 50℃ shaking water bath set at 250 rpm. One ml was drawn at different time periods, centrifuged and the supernant was refrigerated. These samples were analyzed for reducing sugar content by the method of McFeeters with the use of 2.2'-bicinchoninate reagent at 560 nm after appropriate dilution.[19] The enzyme "Cellulosin AP" obtained form Aspergillus niger and "Cellulase Onozuka" from Trichoderma viride were purchased from Ueda Chem. Co and

Yakult Honsho in Japan respectively. The degree of decomposition was determined by the following equation;

degree of decomposition(%)=amount of reducing sugar/weight of substrate×100.

3. RESULTS AND DISCUSSION

3.1 Chemical characteristics

Chemical composition of poplar wood is presented in Table 1. The amount of carbohydrate (holocellulose excepst residual lignin) was almost 80% of the total dry weight while the lignin content was 17.6%. Theses results are in agreement with others[22], although the Klason lignin was in the lower range than was expected for a hardwood. Total lignin content was, however, was in the range of hardwood, considering the content of acid soluble lignin.

Table 1.Chemical composition of *Populus alba*×*P.glandulosa*

Components	%
Extractives*	
Cold water	0.93
Hot water	1.92
Alcohol-cyclo hexane	4.64
1% NaOH	20.65
Holocellulose**	83.23
Residual lignin in holocellulose	2.66
Acid-insoluble lignin(Klason lignin)**	17.60
Acid-soluble lignin	2.61
Ash*	0.58-1.44
pH	6.80

* : percentage based on the dry weight of non-extracted wood.

** : percentage based on the dry weight of extractives free wood.

All values are the averages of 3 replications.

One of the pronounced features was in the relatively higher value of pH(6.80)than that of other woods. High amount of minerals may have contributed to the increase of pH value in this species.

Nutritive value of poplar wood showed that the content of lignin and acid detergent fibre(ADF) was as much high as those of other wood sawdust(Table 2)[15]. Delignification appears, therefore, to be necessary for upgrading the nutritive value for ruminant.

Table 2.Chemical composition of untreated *P.alba*×*P.glandulosa* sawdust as a feedstuff

Neutral detergent fibre (NDF)	88.32
Acid detergent fibre (ADF)	70.06
Cellulose	49.23
Hemicellulose	18.26
Lignin	20.16

Dry matterbasis(%)

Composition of monosaccharides in poplar wood analyzed by HPLC after the hydrolysis with TFA is shown in Table 3. The content of glucose was almost 47% and that of xylose as a major pentose in poplar wood 18%. The amount of hemicellulose, if all the sugars except glucose coudld be regarded as hemicellulose, was 25%, whereas that of pentose about 20%.

Table 3.Sugar composition of *P.alba*×*P.glandulosa*

Sugars	%*
Glucose	46.73
Xylose	18.18
Galactose	3.71
Mannose	1.25
Arabinose	0.40
Rhamnose	0.81

* : percentage based on dry weight of extractives free popular wood.

3.2 Delignfication with alkaline peroxide solution

The results obtained from the delignification of poplar wood with peroxide alkaline solution(pH 11.5) were shown in Figs. 1 and 2. In general the higher the reaction temperature was, the lower the yield of holocellulose was. Because of the increased extraction of hemicellulose and the lignin with increasing the reaction temperature and time, the yield of holocellulose would be decreased correspondingly. The extent of residual lignin decreased in the most reaction conditions except 120°C. The increase of residual lignin in the 120°C may have been caused by the condensation of lignin polymer during the reaction[9]. It was not possible to remove 40-55% of the lignin in the reaction conditions tested in the present experiments like the studies of Gould[12,14]. The

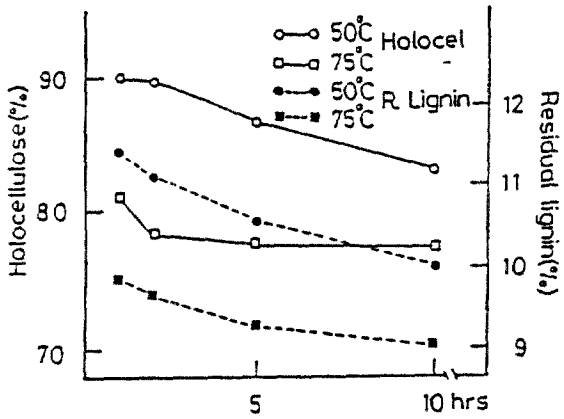

Fig. 1. Change of the yield of holocellulose and the residual lignin during the delignification with alkaline peroxide (1%, pH 11.5) in 50°C and 75°C respectively.

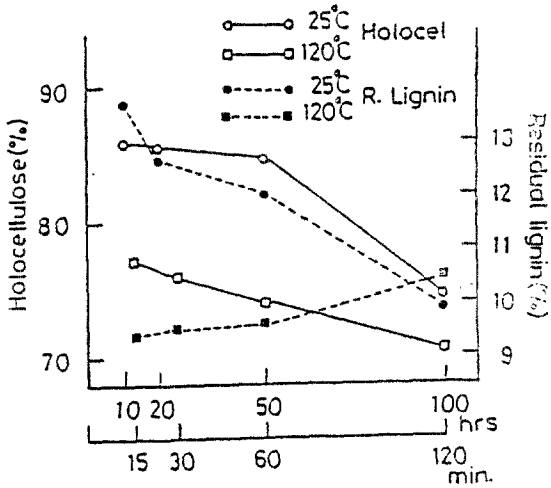

Fig. 2. Change of the yield of holocellulose and the residual lignin during the delignification with alkaline peroxide in 25°C and 120°C respectively.

reason(s) for the resistance of this lignin polymer to alkaline peroxide is not yet clear.

The yield of holocellulose obtained in the reaction conidtion of 25℃/100hrs (74%) is promising because this yield could not be found in the more severed reactions such as 50℃ and 75℃. The same yield as 25℃/100hrs could be obtained only in the very severe condition(120℃/1hr). Over the course of reaction, the pH rose from 11.5 to above 12.0due to the increase in solubilization of carbohydrates. The peroxide was mostly consumed in the reaction of higher temperature(75℃ and 120℃) as shown in Table 4. The concentration of peroxide in the reaction

Table 4.Change of pH value and the concentration of peroxide after the reaction

Reaction conditions		pH value*	H_2O_2 concentration**
Temperature	Reaction time		
	10 hrs.	12.4	0.64
25 °C	20	n.d.	0.61
	50	12.2	0.44
	100	12.3	0.29
	1 hr	11.9	0.53
50 °C	2	12.1	0.40
	5	12.4	0.14
	10	12.4	0.00
	1 hr	12.4	0.00
75 °C	2	12.4	0.00
	5	12.5	0.00
	10	12.8	0.00
	15 min	12.4	0.00
120 °C	30	12.5	0.00
	60	12.5	0.00
	120	12.4	0.00

* : initial pH value was 11.5.
** : initial concentration of peroxide was 0.98-1.04
n.d. : not determined.

of lower temperature also decreased. From the decreased and/or diminished concentration of peroxide during the reaction it can be seen that the removal of lignin during the later part of reaction was not accomplished by the action of peroxide but by sodium hydroxide as the intracrystalline swelling agent which can also affect the alteration in the lignin-carbohydrate complex [20].

All the sawdust of poplar wood pretreated with alkaline peroxide solution showed the higher rate of enzymatic decomposition than that of untreated sawdust(Fig. 3). Nearly all the conditions pretreated showed the two-fold increased in the decomposition rate. Under the extended reaction time of hydrolysis the rate of decomposition in the reaction condition of 25℃/100hrs resulted even in the increase of 350% in comparision with that of the untreated. The depolymerization of cellulose and the splitting the lignin macromolecular by hydrogen peroxide under alkaline

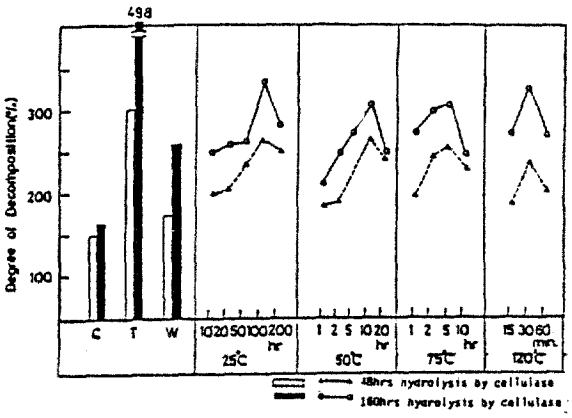

Fig. 3. Effect of the pretreatment on the decomposition of the wood by cellulolytic enzymes (Cellulosin AP + Onozuka R-10,1:1). Wood samples were incubated in citrate buffer (pH 5.0) at 50°C. Degree of decomposition was calculated from reducing sugar divided by weight of substrate. C: non-treated wood sawdust of popular. T: delignified with NaOH and peracetic acid, W: treated with $NaClO_2$ and acetic acid.

condition during delignification may be responsible for the increased hydrtolysis[1,18,27]. The sawdust delignified with $NaClO_2$ and glacial acetic acid[29] showed only two-fold increase in decomposition rate in spite of higher delignification (up to 85%), whereas the holocellulose treated with 1% NaOH solution and 20% peracetic acid successively[26] the five fold increase. From these results it can be seen that the removal of lignin alone does not result in the modification of the crystalline structure required to achieve high hydrolysis rates and yields[17]; increase in the enzymatic susceptibility of the cellulose is not strictly a function of the extent of delignification [23].

The saccharification effiency calculated from the theoretically maximum glucose yield and the measured glucose yield was 19.54% in the non-treated sawdust, while the poplar wood pretreated with 1% peroxide in 25℃/100 hrs showed the highest efficiency(51.24%)among the peroxide treated reactions(Table 5). This result is in agreement with Gould[13] who used white oak(52.5%). However, the sawdust delignified with the method of Toyama and Ogawa recorded

Table 5. Summary of the effect of pretreatment on the saccharification of popula wood

Reaction condition Temp. ℃	Time hr	Rate of delignification (%)	Degree of decomposition* (%)	Saccharification efficiency** (%)
Control (none treated)		0.00	9.13	19.54
Alkaline peroxide (1% H_2O_2,pH 11.5)				
25	100	50.6	31.49	51.24
50	10	50.4	20.56	44.01
75	5	53.8	20.76	46.89
120	30(min.)	52.6	21.87	48.90
Toyama's holocellulose***		100.0	40.19	88.98
Wise'holocellulose****		86.8	17.20	36.83

*: degree of decomposition=reducing sugars produced/weight of substrate.
**: assume native popular wood contains 0.45 g cellulose(=0.467 g glucose) per g(dry weight basis).
***: delignified with 1% NaOH 1hr followed with 20% peracetic acid 1hr.
****: delignified with $NaClO_2$ and glacial acetic acid.

the highest efficiency rate (89%) in the present experiments.

Sugar analysis by HPLC showed that the ratio of glucose to xylose (G/X) in the holocellulose obtained by the method of Wise et al was 2.7-3.0. This ratio is comparable with the non-treated sawdust, while the G/X of the sawdust treated with alkaline peroxide and with the successive treatment of NaOH and peracetic acid showed 4.4 and 4.2 respectively (Fig. 4). The higher ratio of G/X in these conditions due to the rapid degradation of xylose during the delignification reaction can be one of the reasons resulting in the increase of decomposition rate and the saccharification efficiency[28].

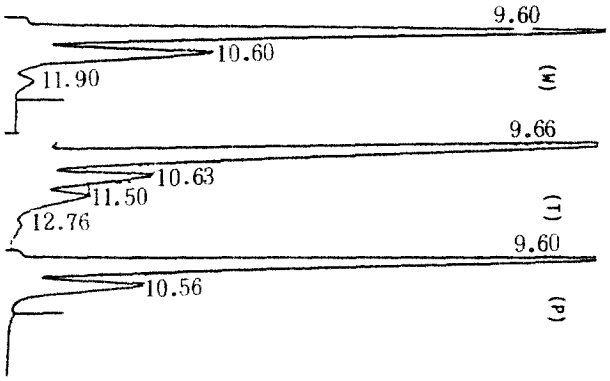

Fig. 4. Composition of sugars delignified with various delignifying agents. W; delignified with $NaClO_2$ and acetic acid by the method of Wise *et al*.T; treated with NaOH and peracetic acid by the method of Toyama and Ogawa.P; reacted with peroxide alkaline solution at 25°C/100 hrs. Analyzed by HPLC (Waters Assoc.) with solvent of acetonitrile: water (85:15). Flow rate 0.5ml/min. Retention time of 9.60 and 11.60 is that of glucose and xylose respectively.

3.3 Selection of optimal delignification condition

For the selection of optimal delignification condition for the enzymatic hydrolysis, the effect of enzymatic susceptibility is more emphasized than the high lignin removal alone. Because even when lignin levels are low, the hydrolysis of cellulose can be limited by the physical properties of the polysaccharide itself like in the delignification with $NaClO_2$ and acetic acid. We regarded the 50% removal of lignin as one of the criterium of optimal condition. Because maximum cellulose degradation occurred only after 50% or more of the lignin has been removed[17,23] and because the removal of only one-third the lignin of hardwoods appears to yield a product equivalent to hay in ruminant digestibility[20]. Furthermore, the rate of decomposition and the saccharification should also be considered.

With these respects it has been found that the pretreatment condition with 1% poeroxide under the condition of 25℃ 100hrs (room temperature) was highly suitable for upgrading the enzymatic hydrolysis. This accerlating the hydrolysis rate was possibly due to the partial removal of lignin and structural swelling of the substrate. However, the reaction condition of 120℃/30 min is also interesting due to its short reaction time, though this condition showed the some what lower value of decomposition rate than that of 25℃/100hrs.

The sawdust treated with the method of Toyama and Ogawa showed the highest rate of saccharification efficiency and decomposition rate. The drawbacks of this reaction, however, are the higher cost of chemicals and energy input. The yield of holocellulose in this pretreatment showed about 70%, which means the considerable losses of non-cellulosic carbohydrates. This process, however, could be adopted only if the high saccharification efficiency is the unique factor to be considered.

Much work remains to be done for this alkaline peroxide treatment to be technically feasible ; rather long reaction time in comparision with other delignification methods, the neutralization of alkali used, the relatively large loss of solubilized hemicelluloses during the delignification and the washing stage and the selection of suitable cellulase systems with high activity.

Further studies are now undertaken for upgrading the nutritive value of this pretreated wood for ruminant animals and for the enhancement of enzymatic hydrolysis by the use of new cellulolytic enzymes[3].

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