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Comparison of Rhizopus Sp. and Lactobacillus Spp. Performance in Lactic Acid Fermentation of Sugar Palm (*Arenga pinnata*) Solid Waste

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Abstract: Lactic acid is an important component of the manufacturing polylactic acid (PLA), which can produce by using high-lignocellulosic biomass (such as rice husk), The sugar palm (*Arenga pinnata*) industry produces an abundant amount of lignocellulosic solid waste. The poor utilization of sugar palm solid waste (SPSW) leads to its potential as a fermentation medium due to its reducing sugar content. Lactic acid fermentation is often conducted using lactic acid bacteria (LAB). However, filamentous fungi such as *Rhizopus sp*. also have the potential to produce lactic acid in fermentation. In the study, SPSW was pretreated using the acid-organosolv method. First, it was ground and screened up to 100-120 mesh. The output sample of the screener was pretreated chemically using dilute acid (H_2SO_4) with a solid-to-liquid ratio of 1:5 w/w for 40 minutes at 120°C and organosolv (ethanol 30%) with a solid: liquid ratio of 1:7 w/w for 33 minutes in 107°C. Enzymatic hydrolysis was performed using the cellulase enzyme. The fermentation was conducted using microorganisms of *Rhizopus sp*. (*R. oryzae and R. arrhizus*) and *Lactobacillus spp.* (*L. casei* and *L. rhamnosus*) at various fermentation temperatures. The fermentation results were analyzed, with the results showing that the lactic acid concentration produced using *Lactobacillus sp*. was higher (4.396 g/L for *L. casei* and 4.089 g/L *L. rhamnosus*) than that using *Rhizopussp*. (2.248 g/L for *R. oryzae* and 2.742g/L for *R. arrhizus*).

Keywords: fermentation, *Lactobacillus spp*, lignocellulose, *Rhizopus sp*, sugar palm solid waste.

乳酸菌优化稻壳生产乳酸的酸催化水解工艺

摘要：乳酸是制造聚乳酸的重要组成部分，可利用高木质纤维素生物质（如稻壳）生产，糖棕（番红花）工业产生大量木质纤维素固体废物。糖棕固体废物利用不佳导致其作为发酵培养基的潜力，因为它的糖含量降低。乳酸发酵通常使用乳酸菌进行。然而，丝状真菌如根霉属。也有可能在发酵中产生乳酸。在该研究中，糖棕固体废物使用酸-有机溶剂法进行预处理。首先，将其研磨并筛选至 100-120 目。使用固液比为 1:5 w/w 的稀酸在 120°C 和固液比为 30% 的有机溶剂（乙醇 30%）对筛选器的输出样品进行化学预处理 40 分钟 1:7 w/w 在 107°C 下持续 33 分钟。使用纤维素酶进行酶水解。使用根霉属微生物进行发酵。（米曲霉和根茎）和乳杆菌属。（干酪乳杆菌和鼠李糖乳杆菌）在不同的发酵温度下。对发酵结果进行分析，结果表明使用乳酸杆菌. 产生的乳酸浓度。比使用根霉属。(米曲霉为 2.248 克/升，根茎为 2.742 克/升)。

关键词：发酵, 乳酸杆菌属, 木质纤维素, 根霉属, 糖棕榈固体废物。

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1. Introduction

The food processing industry generates approximately 45% of the total organic industrial pollution [1]. SPSW is one of the food processing industrial solid wastes produced by the sugar palm flour industry, with a composition of 25.63% cellulose, 12.98% hemicellulose, 29.35% lignin, 1.50% ash, 0.92% moisture content, and 3.98% extractive [2]. The cellulose content can be hydrolyzed into reducing sugar. As well as the cellulose content, the lignin content is also of great concern, as lignin is a recalcitrant substance present in the matrix of cellulose and hemicellulose. Therefore, an initial treatment to reduce the lignin content is needed. Six common pre-treatment processes are used for cellulosic biomass, namely acid pretreatment, alkaline pretreatment, wet oxidation, ionic liquid extraction, oxidative delignification, and organosolv extraction [3]. A study by Lini [4] showed that the combination of acid and organosolv pretreatment successfully increased the efficiency of the delignification process. There are normally two ways to hydrolyze cellulose: chemically and enzymatically. The chemical method uses strong acids, whereas the enzymatic process utilizes a variety of microorganisms [5]. Enzymatic hydrolysis is nevertheless considered the best available procedure because, in contrast to chemical hydrolysis, it does not produce compounds that inhibit the further conversion of the hydrolysate to biofuels and platform chemicals by fermentation [6].

One chemical material for which demand is increasing every year is lactic acid. Global lactic acid production ranges are around 1076.9 tons per year, with an annual growth rate of 14.2% [7]. It can be produced using bacteria and fungi. Lactic acid fermentation using LAB, mainly *Lactobacillus spp*., has been recognized for its ability to convert monomer sugar into lactic acid, with a high product yield and productivity [8]. In a previous study using glucose as the fermentation substrate, *Lactobacillus casei* and *Lactobacillus rhamnosus* were able to produce lactic acid with yields of 0.88 g/g glucose and 0.7 g/g glucose respectively [9, 10].On the other hand, a fungus that has been widely used to produce lactic acid is *Rhizopus sp*. Unlike LAB, Rhizopus strains can grow under low nitrogen sources [11]. Another two important aspects for using fungal lactic acid producer as both enzyme and lactic acid producer, and it may secrete L-lactic acid as the only fermentation product [12]. *Rhizopus arrhizus* and *Rhizopus oryzae* have been proven able to produce lactic acid from starch by fermentation with yields of 0.9 g/g starch and 0.7 g/g starch respectively [13]. To our knowledge, previous research has studied the influence of LAB and filamentous fungus in the fermentation of lactic acid but has not compared the performance of the two microorganisms. This study aims to produce lactic acid from SPSW and observe the differences in lactic acid fermentation using LAB (*L. casei* and *L. rhamnosus*) and fungal microorganisms (*R. oryzae* and *R. arrhizus*).

2. Research Method

The production of lactic acid is shown in Fig. 1 as a graphical method of this research.

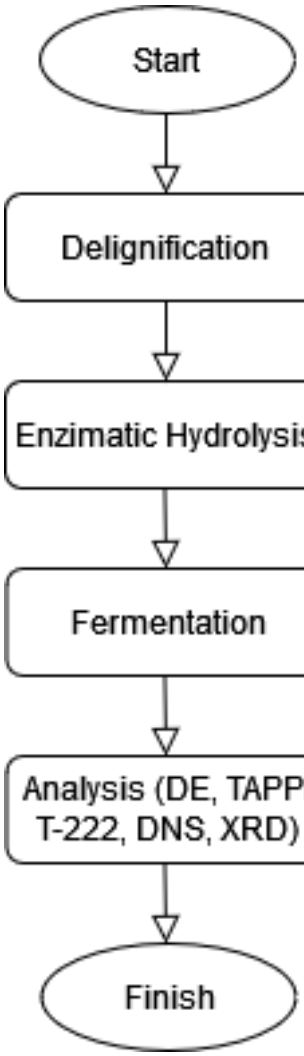

Fig. 1 Graphical method

The experiment is start from delignification, enzymatic hydrolysis, and two steps of fermentation (1st by using Lactobacillus casei + Lactobacillus rhamnosus, and 2nd by using *Rhizopus oryzae and Rhizopus arrhizus*), and the last is the analysis of the result of the experiment. All of them would be explained in detail below.

2.1. Delignification (Pre-treatment of Lignin Reduction)

SPSW was obtained from Tasikmalaya, West Java, Indonesia. It was dried at 60°C and shifted with a screener on 120 mesh. The delignification process involved two main stages. The first was acid pre-treatment. Sulfuric acid 0.2 M was added to SPSW with a solid-to-liquid ratio of 1:5 w/w. Next, a second stage was conducted usingorganosolv pretreatment to remove the lignin content. Ethanol 30% and NaOH 3% as a catalyst with a solid-to-liquid ratio of 1:7 were

added to SPSW with acid pre-treatment. The reaction between the solid residues and the organosolv took place on an autoclave at 107°C for 33 minutes [2].

2.2. Enzymatic Hydrolysis

An enzymatic hydrolysis process was used to obtain reducing sugar from the SPSW. 2 g of pretreated SPSW was added in an Erlenmeyer flask and enzymatic hydrolysis was performed using commercial cellulase from *Trichoderma reseei*. For the reactions, a cellulase loading of 0.931 U/ml was added to a sodium citrate buffer (pH 5.5) of 60 ml. The process was performed in an incubator shaker at 60°C, 125 rpm, and for 24 hours.

2.3. Fermentation Using Lactobacillus Casei and Lactobacillus Rhamnosus

MRS was used as the pre-culture medium. Loopful of cells was added to the medium then incubated for 12 hours at 37°C [14]. The nitrogen source for the fermentation process was made using 10 g/l $(NH_4)_2SO_4$ and 2.5 g/L yeast extract [15]. The composition of inorganic salts was (g/l): (0.05) $MnSO_4.7H_2O$, (0.2) $MgSO4.7H_2O$, and (0.5) KH_2PO_4. 10 g/l of $CaCO_3$ was added as a neutralizing agent. Fermentation was performed in an incubator shaker at 150 rpm, with an initial pH of 5.5, and for 48 hours.

2.4. Fermentation Using Rhizopus Oryzae and Rhizopus Arrhizus

The medium was inoculated with a spore suspension containing 10^5 spore/ml. It was then incubated at 30°C in an incubator shaker at 150 rpm for 12 hours [16]. The hydrolysate from the enzymatic hydrolysis was centrifuged at 4°C, 10000 rpm, and for 10 minutes. The fermentation substrate consisted of (g/l): (1) $(NH_4)_2SO_4$, (0.38) $MgSO_4.7H_2O$, (0.1) $ZnSO_4.7H_2O$, and (0.15) KH_2PO_4 as inorganic salt and nitrogen sources. The fermentations were performed in Erlenmeyer flasks, each containing hydrolysate from the enzymatic hydrolysis process. The initial pH of the fermentation process was 6.5. Fermentation was performed in an incubator shaker at 150 rpm for 48 hours [16].

2.5. Analytical Method

The cellulose, hemicellulose, and lignin content in the SPSW was calculated using DE and TAPPI T-222, while the reducing sugar content was analyzed using DNS methods. The concentration of lactic acid was quantified according to the colorimetric method of Barker and Summerson [17].

XRD analysis was used to obtain the crystallinity index (CrI). This was calculated from the following equation:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100\% \quad (1)$$

3. Results and Discussions

2.1. Delignification and Enzymatic Hydrolysis

SPSW was used for fermentation substrate, with treatment needed using acid-organosolv and enzymatic hydrolysis. Before the delignification process, the amount of cellulose was (%, dry weight basis) ~40%, hemicellulose ~16% and lignin ~27%. Acid-organosolv was used to remove the lignin, which is an undesirable component, besides being a physical barrier for enzymes [18]. After treatment with acid-organosolv, the SPSW contained (%, dry weight basis) ~62% cellulose, ~10% hemicellulose, and ~19% lignin. The lignin and hemicellulose content, therefore, decreased, while that of cellulose increased. This result was confirmed by XRD analysis of the SPSW sample before and after pretreatment; Fig. 2 shows the increasing CrI parameter. This result shows I_{002} as a crystalline fraction in position $2\Theta = 22^0$ and I_{am} as an amorphous fraction in position $2\Theta = 18.7^0$ [4].

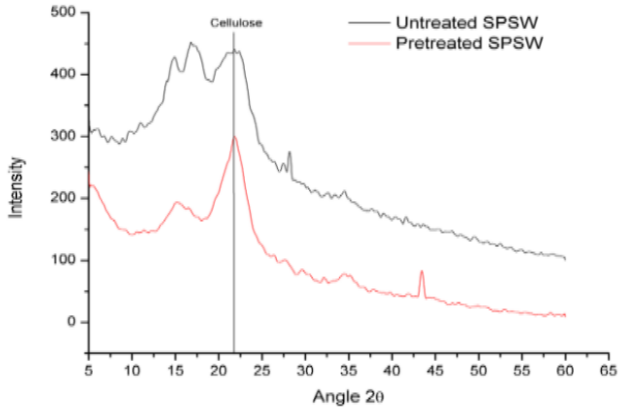

Fig. 2 XRD analysis results in SPSW before and after pretreatment

The increased CrI after delignification meant that the delignification process had damaged the glycosidic bond and removed the amorphous structure of the hemicellulose and lignin. Based on the study by Park [19] the amorphous structure is more easily digested by enzymes.

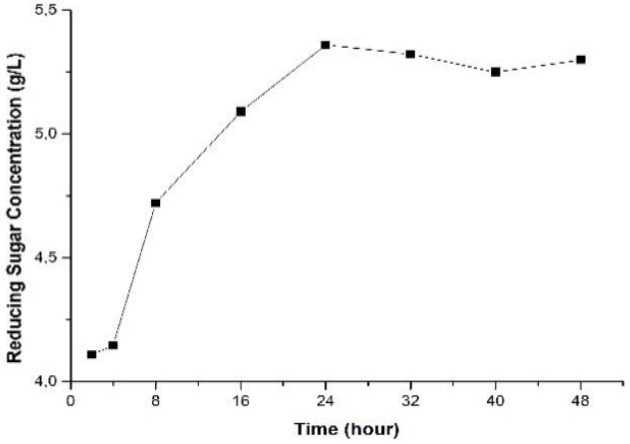

Fig.3 Reducing sugar concentration during enzymatic hydrolysis

Fig. 3 shows the profile of reducing sugar reduction from the enzymatic hydrolysis of SPSW. The reducing

sugar concentration increased over time and reached maximum levels after 24 hours. The reducing sugar produced from the enzymatic hydrolysis was used as the main substrate for fermentation.

2.2. Effect of Temperature

2.2.1. Effect on Lactobacillus spp.

L. casei and *L. rhamnosus* were adapted to four temperatures (29, 33, 37, and 41^{o}C). Lactic acid production increased with the rise in temperature up to 37^{o}C, however, a significant decrease in its concentration was found at 40^{o}C, as seen in Table. 1. The highest concentration of lactic acid was 4.396 g/L in *L. casei* at a temperature of 37^{o}C. A slightly lower concentration of 4.089 g/L was obtained by *L. rhamnosus* at the same temperature.

Table 1 Lactic acid concentration by *Lactobacillus spp.*

Temperature (^{o}C)	The concentration of lactic acid (g/L)	
	L. casei	***L. rhamnosus***
29	2.343	1.475
33	3.674	2.686
37	4.396	4.089
41	1.657	1.059

These results conform with the experiments conducted by Hujanen [20], which found that the best temperature for the production of *L. casei* and *L. rhamnosus*was 37^{o}C, with a maximum concentration of 80 g/L by *L. casei* and 70 g/L by *L. rhamnosus*. Another study also determined the optimum temperature for lactic acid fermentation by *L. casei* to be 37^{o}C, with a maximum concentration of 44.88 g/L [21]. A further study reported that a temperature of 37^{o}C gave the highest concentration of 20.34 g/L in lactic acid production by *L. rhamnosus* [22]. In this experiment, *L. casei* was able to produce a higher lactic acid concentration than *L. rhamnosus* at all temperatures, as seen in Table 1.

3.2.1. Effect on Rhizopus Sp.

*R. oryzae*and *R. arrhizus* were adapted to four temperatures (30, 34, 38, and 42^{o}C) based on a previous study conducted by [16]; fermentation using *Rhizopus sp* at temperatures below 30°C produces low lactic acid concentration. *Rhizopus* sp. gave different lactic acid concentration results compared to *Lactobacillus* sp. The best concentration of lactic acid with *R. oryzae* was at a temperature of 30°C, with a concentration of 2.1423 g/L (see Table 2). There were no significantly different results at the temperature of 34°C or 38°C, while at 42°C the concentration of lactic acid decreased because *R. oryzae* cannot grow at high temperatures. This is consistent with a study conducted by [16], in which the best production of lactic acid by *R. oryzae* was at 30°C, while at 45°C no lactic acid was produced. In addition, a study conducted by [23] also concluded that a temperature of 30°C was optimum for the fermentation process using *Rhizopus sp.*

Table 2 Lactic acid concentration by *Rhizopus sp.*

Temperature (^{o}C)	The concentration of lactic acid (g/L)	
	R. oryzae	***R. arrhizus***
30	2.248	2.743
34	2.239	2.514
38	2.232	2.528
42	1.717	1.670

Regarding the influence of temperature on fermentation using *R. arrhizus*, the best result was also obtained at a temperature of 30°C, namely 2.743 g/L. However, the concentration of lactic acid decreased with increasing temperature. This is in line with the research conducted by Huang [24], which found that that *R. arrhizus* can grow at temperatures of 22-38 °C.

2.3 Effect of microorganism type in optimized temperature fermentation

Temperature is one of the important factors which influences the activity of metabolic/cell enzymes. Enzymes are most active at an optimum temperature and the enzymatic reaction proceeds at a maximum rate. However, below and above the optimal temperature the reaction rate decreases, which causes a problem in cell metabolism [14].

Table 3 Comparison of lactic acid concentration by *Lactobacillus spp.* and *Rhizopus sp.*

Species	The concentration of reducing sugar (g/L)	Yield (g lactic acid/g reducing sugar)
L. Casei	5.376	64,23%
L. Rhamnosus	5.376	59,74%
R. oryzae	5.361	32.85%
R. arrhizus	5.361	40.08%

The highest yield was obtained by *L.casei* in the *Lactobacillus* strain and *R.arrhizus* in the *Rhizopus* strain. However, *Rhizhopus* strains produce lower yields than *Lactobacillus* sp, as shown in Table 3. Overall, the yield of lactic acid produced in this study was average compared to other studies. A study by Senedese [25] yielded 0.446 g lactic acid/g glucose using *Lactobacillus spp.* bacteria, while another study conducted by Jin [13] yielded 0.65 – 0.76 g lactic acid/g glucose using *Rhizopus spp*. The lower yield of lactic acid produced was mostly influenced by the low initial glucose concentration. Acid fermentation using *Lactobacillus*spp. has a theoretical maximum yield of 2 mol lactic acid/mol glucose by homolactic fermentation in anaerobic conditions (EMP pathway) [8], while *Rhizopus* only yields 1.5 mol lactic acid/mol glucose [26]. The EMP pathway converts glucose into lactic acid based on the following reaction equation [8]:

$$Glucose + 2Pi + 2ADP \rightarrow 2LA + 2ATP + 2H_2O \quad (2)$$

In this experiment, *L. casei* was able to produce a higher lactic acid concentration than *L. rhamnosus* at

all temperatures. This result is also confirmed by the glucose consumption and microbial growth of *L. casei*, which is greater than that of *L. rhamnosus*, as seen in Fig. 4 The higher growth of bacteria in *L. casei* compared to *L. rhamnosus* is in line with the higher lactic acid concentration produced in fermentation.

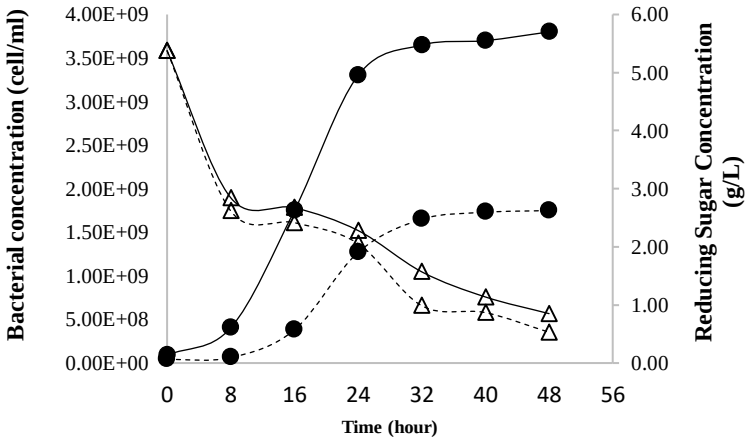

Fig. 4 Effect of bacterial type on the fermentation process. ● represents microbial growth; Δglucoe consumption; –*L.casei*; - - *L.rhamnosus*

However, the increase in lactic acid concentration by *R. arrhizus* was not followed by microbial growth. According to Fig. 4, *R. oryzae* has greater microbial growth than *R. arrhizus* during the fermentation process. The study conducted by Jin [13] showed that *R. oryzae* had cell growth of 4.8-5.3 g/l, whereas *R. arrhizus* had lower cell growth of 2.2-2.4 g / l, according to the results of lactic acid production followed by the growth phase. This shows the importance of competition in using carbon sources for biomass formation and lactic acid production in the fermentation process [23]. Decreased glucose levels also occur in *R. oryzae* and *R. arrhizus*. Glucose is the main monosaccharide in hydrolyzate, and with the cultivation of *R. oryzae* the substrate will be consumed and the product will be formed [27].

This study showed a relatively low concentration of lactic acid produced. This low concentration might have been the result of multiple factors, such as pH, nutrient concentration, substrate concentration, and temperature [28]. In this study, the factor which influenced the low concentration most was substrate concentration. The initial glucose concentration used was sufficient to produce a higher lactic acid concentration.

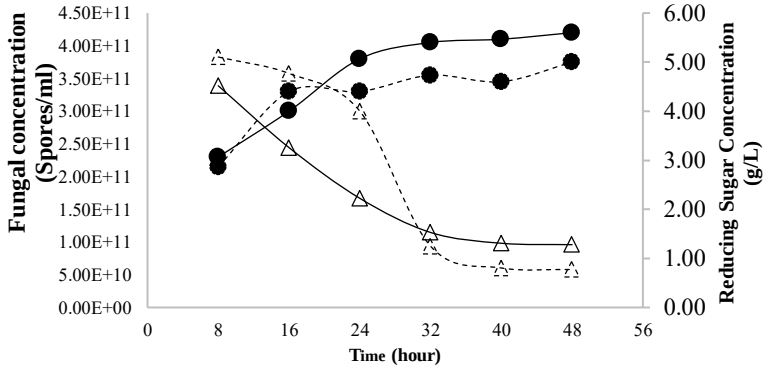

Fig.5 Effect of fungal type on the fermentation process. ● represents microbial growth; ▵ glucose consumption; ― *R.oryzae*; - - *R. arrhizus*

Lactic acid-producing bacteria include the wild type and engineered producers. In general, bacterial lactic acid fermentation suffers from several limitations, including (i) production of both L- and D- lactic acid; (ii) low yield due to byproduct formation; (iii) use of a nutritionally-rich medium; and (iv) a high risk of bacteriophage infection, which results in cell lysis and subsequent cessation of lactic acid production [29]. Limitations (i) and (ii) can be solved by using a specific type of *Lactobacilli* species which only ferments glucose into lactic acid with high optical purity. These bacteria include *L. casei* and *L. rhamnosus* [8].

Even though they produce a lower yield, *Rhizopus* strains have some advantages compared to lactic-acid producing bacteria, including their low nutrient requirements [30]; a low-cost downstream process due to their filamentous or pellet growth that makes separation from the fermentation broth easier than that of bacteria or yeast [31]; and production of fungal biomass as a valuable fermentation byproduct [29].

4. Conclusions

SPSW can be delignification successfully to remove lignin content with the amount of lignin in SPSW decreasing to 19%. It has been proven that SPSW can

be converted to lactic acid through the fermentation process with *Lactobacillus* spp. and *Rhizopus* sp. The highest lactic acid production was obtained using *Lactobacillusspp*. The yield of lactic acid from the fermentation process with *L.casei* and *L.rhamnosus*was 64.23% and 59.74% respectively, while the yield of lactic acid using *R.oryzae* and *R.arrhizus* was lower, at 32.85% and 40.08% respectively. Each value was obtained from the optimum temperature, which was 37°C for *L.casei* and *L.rhamnosus* and 30°C for *R.oryzae* and *R.arrhizus*. The highest yield of lactic acid was obtained by *L. casei*, at 63.24%. This study showed a relatively low concentration of lactic acid produced. Such a low concentration might have been the result of multiple factors, such as pH, nutrient concentration, substrate concentration, and temperature. In this study, the factor which influenced the low concentration most was substrate concentration. The initial glucose concentration used was sufficient to produce a higher lactic acid concentration. Even though they produce a lower yield, all of the strains have some advantages compared to lactic-acid producing bacteria, including their low nutrient requirements; a low-cost downstream process due to their filamentous or pellet growth that makes separation from the fermentation broth easier than that of bacteria or yeast; and production of fungal biomass as a valuable fermentation byproduct. For the next study should do optimization using a statistical method like Response Surface Methodology (RSM) to reach the optimum value for the best result of an experiment.

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