Original Research

Decolorization of Humic Acids and Alkaline Lignin Derivative by an Anamorphic *Bjerkandera Adusta* R59 Strain Isolated from Soil

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Abstract

An anamorphic *Bjerkandera adusta* R59 strain, isolated from soil, was found to decolorize post-industrial lignin alkaline fraction, humic acids isolated from two kinds of soil and from brown coal. The drop of methoxyphenolic compound levels in liquid *B. adusta* cultures containing lignin or humic acids was correlated with decolorization of studied biopolymers, which suggests their partial biodegradation. It was shown that this process was coupled with the induction of secondary metabolism (idiophase), and highest peroxidase activity in culture medium and appearance of aerial mycelium. Decolorization of lignin and humic acids from lessive soil and brown coal depended on glucose presence (cometabolism). Decolorization of humic acid from chernozem was related partially to adsorption by fungal mycelium.

Keywords: anamorphic fungus, decolorization, humic acids, lignin

Introduction

Pollution of drinking waters by dyes or other colorizing substances is a serious problem because many of them exhibit toxic, mutagenic or carcinogenic properties or may be transformed into compounds possessing such properties [1, 2]. These substances leak into the water reservoirs naturally (e.g. humus compounds) or from inadequately purified post-industrial wastes. Humus substances naturally polluting fresh waters include mainly humic and fulvic acids, and their concentration in Miocene groundwaters may exceed 100 mg ml^{-1} [3-5]. Also, the toxic and biodegradation-resistant products of the paper industry may increase the pollution of water. These products include lignin and its derivatives: alkaline lignin, thiolignin and lignosulphonates [6]. The pharmaceutical industry also contributes to the general pollution; it releases to the environment post-production wastes rich in anthraquinonic antibiotics, e.g. daunomycin – a cytostatic drug revealing high affinity to nucleic acids [7].

Traditional methods of colored substance removal from drinking waters and wastewaters are generally based on chemical decolorization (chlorination), but this does not effectively solve the problem of wastewater utilization. The lignin derivatives remain in the chlorinated waters in altered forms: as highly toxic, mutagenic and carcinogenic chlorolignins, chlorophenols, chloroguaiacols and dioxins [2]. Chlorination of humic substance-containing waters also results in the formation of toxic, carcinogenic trihalomethans e.g. chloroform and haloacetic acids, acetonitriles, chloropicrin and

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3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone − a main mutagenic factor in chlorinated water [3, 5].

Biological methods of water purification, especially those based on biosorption and microbial biodegradation, are postulated to be safer than the chemical ones, mainly because the toxicity of by-products of biological methods is minimized [8]. Moreover, these methods are attractive for their low costs, environmental safety and common society acceptance [1].

The alternative methods proposed for decolorization of polluted drinking water are based on the use of white-rot fungi, synthesizing exogenous oxidoreductases: oxygen-dependent laccase and H_2O_2-dependent peroxidases. These enzymes catalyze the oxidative depolymerization of lignin in lignocellulose wood complex [9] but also of other aromatic compounds possessing a lignin-like structure. Among white-rot basidiomycete the *Panerochaete chrysosporium* strain revealed the greatest effectiveness at decolorization and biodegradation of lignosulphonates, Kraft lignin and humic acids. Practical application of this strain in decolorization of Kraft lignin-rich post-production wastewaters was also reported [6, 8].

Decolorization and biodegradation abilities of different *Bjerkandera* sp. including *B. adusta* strains were widely studied [10, 11, 12]. A new strain of this fungus (*B. adusta* R59), isolated from soil was recently described [13]. *B. adusta* R59 strain is the imperfect (anamorphic) stadium of the fungus, similar to *Geotrichum* (mitosporic fungi). This feature distinguishes *B. adusta* R59 from already reported and described [11, 14] perfect *B. adusta* strains (Basidiomycetes). This strain performed the decolorization of diluted but also non-diluted (after the fungus adaptation) daunomycin-containing post-production effluent due to its partial biodegradation [13].

This work presents the results of our pilot laboratory experiments concerning the decolorization of lignin and humic acids by the *B. adusta* R59 strain.

Materials and Methods

Fungal Strain

Anamorphic (mitosporic fungi) *B. adusta* R59 strain, was isolated from the black earth soil (Pheozems, FAO classification) from a field near Lublin (southeastern Poland). Isolation procedure and identification of the R59 strain was described elsewhere [13]. Nucleotide sequences (rRNA gene fragment) of the fungus are available in GeneBank under the accession number AY 319191 (http://www.ncbi.nlm.nih.gov). The strain has been deposited in the CCBAS culture collection under the accession number CCBAS930 (http://www.biomed.cas.cz//ccbas/fungi.htm).

Source of Lignin and Humic Acids

Lignin (LG), precipitated from first alkaline fraction (from wood extract) by acidification with H_2SO_4, was obtained from InterCell S.A. (Ostrołęka, Poland). Before use, the lignin was dissolved in sterile 0.1 M NaOH and adjusted to pH 7.0 with 0.2 M HCl. The lignin contained (g/kg of dry weight): 408.2 of carbon, 40.4 of hydrogen, 0.2 of nitrogen; no ashes.

Humic acids (HAs) were obtained from brown coal (Fluka, Switzerland) or isolated from chernozem and lessive soil according to Schnitzer and Schuppli [15].

Conditions of *B. Adusta* R59 Growth

Pilot experiments of lignin and humic acids decolorization were performed on Petri dishes (ø=9 cm) with Park and Robinson agar medium [16] with 0,25% glucose and 0.2% lignin, 0.03% appropriate humic acid. The plates were inoculated by round pieces (ø=1 cm) of R59 strain mycelium after 7 days of growth on potato-glucose (20% potato, 2% glucose) agar medium. Abilities of *B. adusta* strain to decolorize LG, HAs were estimated on a base of the extent of decolorization spheres (% of a total medium surface) [17].

Decolorization of lignin and humic acids was tested also in liquid cultures under stationary conditions. 100 ml Erlenmeyer flasks were filled with 50 ml of either mineral medium for ligninolytic fungi [18] enriched with 0.2% lignin or Park and Robinson [16] medium containing 0.01% humic acids. In each case, the media were supplemented with 0.25% glucose. All versions of media were inoculated with 1 ml of homogenized *B. adusta* R59 mycelium (10^5 cfu ml^{-1}) from a 7-day-old culture of fungus on potato-glucose liquid medium. Non-inoculated media served as controls. Cultures and controls were incubated at 26ºC up to 60 days. The experiment was made in triplicate.

Analytical Methods

In liquid *B. adusta* R59 cultures enriched with LG, HAs, the following parameters were estimated:

- decolorization rate: for LG at 430 nm (maximum absorbance), for HAs at 400 nm [19]
- peroxidase activity according to Maehly and Chance [20] method with o-dianisidine as a substrate (previous optimization showed that *o*-dianisidine was the best substrate for *B. adusta* R59 peroxidase)
- methoxyphenolic compounds concentration was determined at 500 nm (for vanillic acid detection) according to Malarczyk [21]
- amount of HAs absorbed by fungal mycelium according to Dari [22]. HAs absorbed by mycelium were eluted with 0.2M NaOH for 24h at room temperature and estimated at 400 nm at the same pH and volume parameters as at the beginning of the experiment
- pH of medium
- All assays were performed in triplicate.

Observations of Cultures

During culturing, the macroscopic and microscopic observations of mycelium growth and development were performed. Type of growth, sporulation, ratio of visible decolorization of medium and color of mycelium were estimated.

Table 1. Decolorization (%) of 0.2% LG and 0.03% HAs on agarized Park and Robinson medium by *B. adusta* R59.

Substrate	Time of culture (days)				
	3 (5*)	7	10	14	18
LG	20	33	65	78	100
HAs from brown coal	28	44	55	100	100
HAs from lessive soil	22	39	50	78	100
HAs from chernozem	0	0	22	50	100

* – for LG

Evaluation of Results

Results were analyzed by the statistical method counting the standard deviations. Correlation coefficients (r) between decolorization degree and phenolics level in liquid culture media were estimated.

Results and Discussion

Decolorization of Lignin and Humic Acids on Agarized and in Liquid Media by *B. Adusta* R59

Pilot experiments on decolorization of tested toxic substances by *B. adusta* R59 were performed on agarized medium. Decolorization of 0.2% LG on Petri dishes with agarized medium began on the 5th day of the experiment, while total decolorization (correlated with the total overgrowth of plates by R59 mycelium) was observed after 18 days of *B. adusta* growth (Table 1). In an analogous experiment with 0.03% humic acids, the decolorization also lasted up to 18 days, with a rate dependent on HAs source. The beginning of the process (in the case of HAs from lessive soil and brown coal) was observed on the 3rd day of R59 growth. Total decolorization of medium and simultaneous total overgrowth of plates by R59 mycelium were found on the 14th or 18th day of *B. adusta* growth, respectively (Table 1). The results confirm our previously presented data [17] concerning the partial decolorization of HAs.

Our results confirmed the data obtained by other researchers. Moreira et al., [14] reported that *Bjerkandera adusta* is highly effective at decolorization of lignin-rich post-production effluent in stationary cultures. Gramss et al. [23] showed that this fungus decolorized brown solutions of humic acids.

Removal of LG and HAs from liquid cultures proceeded much slower than in the case of solid media. Visible decolorization of 0.2% alkaline LG was the slowest, with the beginning at the 17th day of culturing, and completed after 25 days of R59 strain growth, with a correlation with 33% decrease of medium absorbance in comparison with its initial value. The final effect of alkaline lignin decolorization after 60 days of culture corresponded to a 50% reduction of absorbance (Fig. 1A), leaving the post-culture medium slightly yellowish.

The decolorizing properties of *B. adusta* R59 strain in the case of lignin fractions are relatively weak when compared with *Phanerochaete chrysosporium* which is able to decolorize 0.09% alkaline lignin solutions (black liquor) in 60% during just 7 days [24]. This result, however, was obtained using *P. chrysosporium* mycelium immobilized on porous plastic rings. In our research we also found that R59 mycelium in solid cultures decolorized lignin more effectively (65% during 10 days; Table 1) than in liquid cultures (Fig. 1A). In natural conditions, ligninolytic fungi are adapted to decompose the lignin in solid form (wood fragments, soil); this may explain higher lignin-decaying effectiveness of ligninolytic fungi on solid than in liquid media.

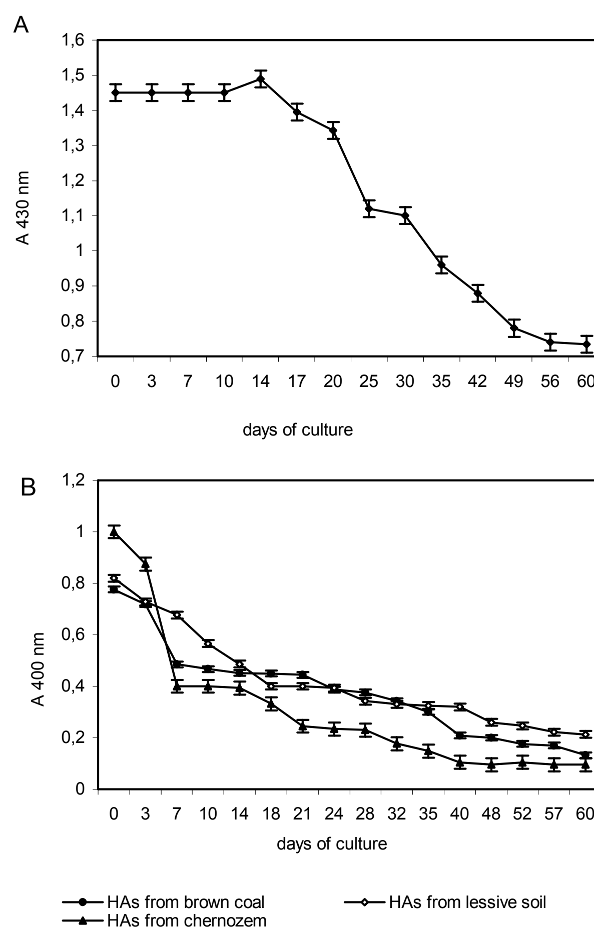

Fig. 1. Decolorization of: 0.2% lignin (A); 0.01% humic acids (B) by *B. adusta* R59 strain.

Table 2. Adsorption of HAs from chernozem after 30 days of *B. adusta* R59 growth on liquid medium.

Initial concentration of HAs	mg of HAs		(a + b) HAs not degraded by mycelium	Adsorption (%)
	Remaining in medium (a)	Adsorbed by mycelium (b)		
0.01% (5.0 mg)	3.815*	0.314	4.129	6.28
	3.717	0.233	3.95	4.66
	3.668	0.340	4.008	6.8
	3.886	0.240	4.126	4.8

* - repetitions

In the case of 0.01% humic acids in liquid media, the beginning of decolorization appeared on the 7th day of R59 growth. The decolorization of media containing HAs from chernozem, brown coal and lessive soil corresponded to 60, 38 and 17% loss of their initial absorbance, respectively (Fig. 1B). Thereafter, decolorization of the media augmented steadily and after 60 days it finally reached the values of 90.5% and 74% for HAs from lessive soil and brown coal, respectively (Fig. 1B). These values are comparable with HAs decolorization results reported by Gramss et al. [23], who described the 41-73% decolorization of these compounds (initial concentration: 0.08%) within 3 weeks by white-rot fungi. However, it seems that decolorization of 0.03% humic acids by R59 strain proceeds more effectively when performed on agarized media than in the liquid ones (Table 1).

In the case of 0.01% HAs from chernozem, total visible decolorization (data not shown) was observed on the 7th day of R59 growth (data not shown). This phenomenon was accompanied by 60% absorbance decrease (Fig. 1B) and partial HAs absorption by mycelium. However, the amount of absorbed HAs was relatively low and even after 30 days of the experiment did not exceed 6% of total HAs (Table 2). This phenomenon did not appear in the case of HAs from lessive soil and brown coal. Similar results were reported in the case of *Rhizopus arrhizus* (Zygomycota) cultures, where it was postulated that microbial cell wall (especially its chitin components) played a crucial role in the process of humic acid absorption [25]. However, in the case of *B. adusta* R59 strain, weak absorption (~5.5% of total HA) does not seem to be of great significance. These results confirm the reports of other authors. Poiting and Vrijmoed [26] showed that the amount of azo dyes and triphenylmethane dyes absorbed by *Phanerochaete chrysosporium* mycelium did not exceed 3%. Other authors [27] reported that the amount of colorizing substances absorbed by fungal mycelium may reach even 40%, but only in cases of shaken liquid cultures.

Growth of *B. Adusta* R59 Strain and pH Changes in Cultures with LG and HAs

Decolorization of LG and HAs from lessive soil and brown coal in liquid media depended on the formation of aerial mycelium (Table 3). In these media, the total overgrowth of medium surface by sporulating aerial mycelium observed after 20 days (in case of LG) or 30 days (HAs from lessive soil and brown coal) of fungal growth was correlated with visible medium decolorization. Moreover, media with HAs from lessive soil and brown coal and also with LG were acidified by 1-1.5 units (to pH 5.05-5.3), while in media supplemented with dyes, the pH dropped quickly to 3.0-3.5 and then increased to 5.0-6.0 (Table 3).

Changes in Peroxidase Activity and Phenolics Level in Culture Media Containing LG and HAs

Activity of peroxidase in liquid culture medium containing LG and HAs appeared on the 3rd day of *B. adusta* growth (Fig. 2A, B). Its activity, in the case of medium

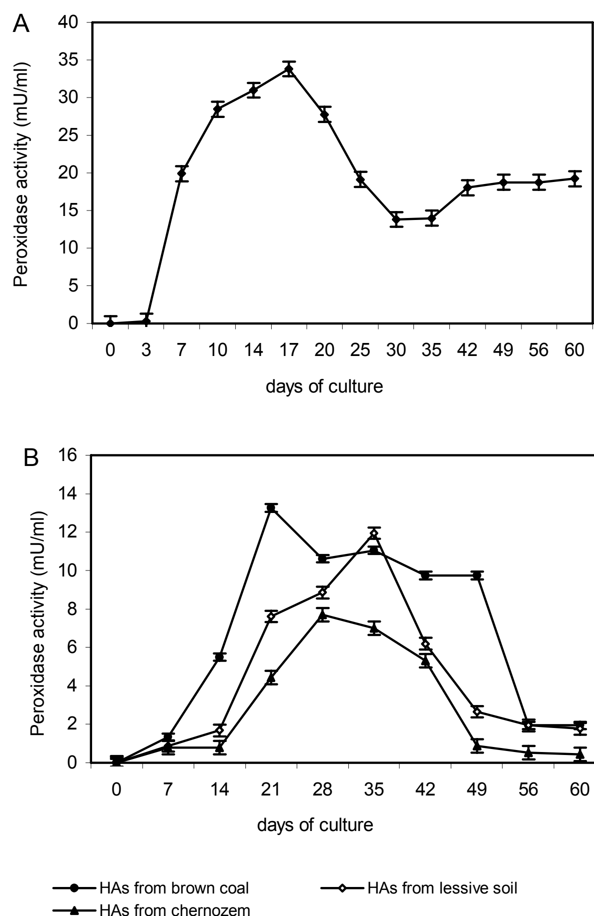

Fig. 2. Peroxidase activity in liquid cultures of *B. adusta* R59 containing: 0.2% lignin (A); 0.01% humic acids (B).

Table 3. Growth and pH changes in liquid cultures *B. adusta* R59 with 0.2% LG and 0.01% HAs.

Days	LG		HAs					
			Brown coal		Lessive soil		Chernozem	
	g	pH	g	pH	g	pH	g	pH
0	-	6.54	-	6.51	-	6.43	-	6.40
10	+	5.58	+	5.13	+	5.24	+	5.44
20	+++	5.05	++	5.20	++	5.30	+	5.15
30	+++	5.07	+++	5.32	+++	5.20	++	5.29

"g" – growth;
"-" – lack of growth of vegetative mycelium and slight presence of aerial mycelium;
"+++" – significant growth of vegetative and aerial mycelia

containing LG, reached the maximum (33.81 mU ml^{-1}) on the 18th day of R59 growth simultaneously with the beginning of aerial mycelium formation (Table 3); afterwards, enzyme activity dropped to half of its maximum activity (on 25th day) simultaneously with the moment of total visible decolorization of the medium (Fig. 2A, Fig. 1A). In case of the media containing HAs, the highest peroxidase activity was found in versions with HAs isolated from brown coal (13.27 mU ml^{-1} at 21st day of culture). Maximum activities of peroxidase in media containing HAs from lessive soil and from chernozem were lower (11.95 and 7.7 mU ml^{-1}, respectively) and appeared later (at 32nd and 28th day of culture, respectively). At the end of the experiment, the enzyme activities dropped to values less than 10% of its maximum value (Fig. 2B), which was correlated with almost total decolorization of the medium (Fig. 1B). Therefore, we generally concluded that extracellular peroxidase activity is important for decolorization of lignin and humic acids tested in our work. Numerous authors, such as Gramss et al. [23] and Moreira et al. [14], postulated the crucial role of peroxidase in decolorization and biodegradation of lignin and humic acids. Peroxidase activity was also found in cultures *B. adusta* R59 grown in the presence of humic acids, lignocellulose [17] and daunomycin [13]. Activity of laccase, other model ligninolytic enzyme of white-rot fungi system, was not detected in *B. adusta* R59 cultures containing lignin (data not shown). However, in our previous study, laccase was detected exclusively in cultures enriched with humic acids extracted from brown coal [17]. Such a low laccase activity with simultaneous high activities of peroxidase was already reported in the case of Kraft lignin [14] decolorization by *B. adusta*. Moreira et al. [14], on the basis of results of fungal screening, postulated that active lignin decolorization may be performed only by fungi exhibiting low laccase but high peroxidase activity.

Phenolics (methoxyphenolics groups) level in media containing LG and HAs decreased steadily since the 3rd day of *B. adusta* growth, with the exception of HA from lessive soil, where this process started on the 14th day of culture (Fig. 3A, B). Degradation of these substances was more effective in media containing LG (50% of initial amount) than HAs (15–25% of initial amount) (Fig. 3A, B). Methoxyphenolics amounts dropped at the end of the experiment (60 days) to ~25% of their initial value for medium with LG and to ~60-70% of their initial values for versions with HAs.

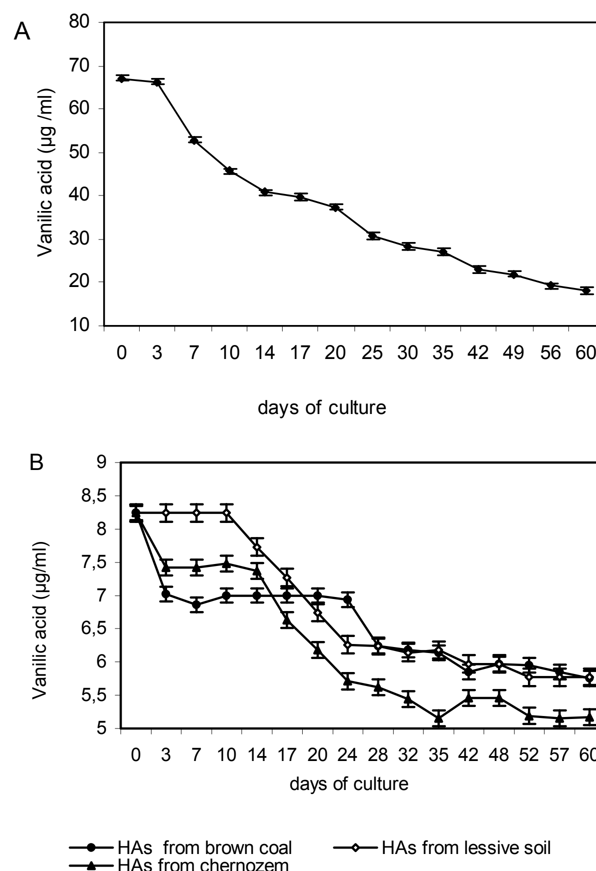

Fig. 3. Content of methoxyphenolic compounds in liquid cultures of *B. adusta* R59 containing: 0.2% lignin (A); 0.01% humic acids (B).

Decrease of methoxyphenolics level (result of demethylation of these groups) in tested media containing lignin and humic acids from brown coal, lessive soil and chernozem was highly correlated with decolorization level (r = 0.880, 0.977, 0.956 and 0.900, respectively, with α=0.001), suggesting that these compounds were biodegraded. Similar results were obtained in *B. adusta* R59 cultures enriched with daunomycin [13]. Demethylation of methoxyphenolic groups in lignin is, according to Leonowicz [9], a peroxidase-catalyzed starting point of lignin biodegradation in white-rot fungi cultures.

In our earlier research [13] we showed that biodegradation of daunomycin by *B. adusta* R59 strain appeared in idiophase (secondary metabolism). Similar observations were found for the substrates studied in this article. Maximum peroxidase activity was observed in the cultures simultaneously with LG and HAs decolorization and with aerial mycelium formation by R59 strain. The trophophase-idiophase shift of fungal metabolism, manifested by the appearance of aerial mycelium, is equal to the shift to a secondary metabolism phase. Lignin biodegradation by an anamorphic form of *B. adusta* R59 is therefore a process dependent on fungal secondary metabolism. This statement is in agreement with current knowledge concerning the lignin metabolism of white-rot fungi.

Lignin cannot be degraded and serve as a sole carbon and energy source for fungi; ligninolysis occurs only when other readily biodegradable substrates are available [28]. Participation of microbial extracellular peroxidase in the humic acid decolorization process and the necessity of glucose presence in the culture medium were already reported [22]. We came to similar conclusions on the basis of results observed for *B. adusta* R59 strain, presented in this work. In medium without glucose (data not shown), the decolorization of tested substances was inhibited and the fungus did not produce aerial mycelium, which suggests repression of secondary metabolism pathways. The fact that decolorization of lignin and humic acids was observed only in the presence of glucose postulates that this process performed by *B. adusta* R59 cultures is cometabolic in its character.

Conclusions

Results obtained in our experiments lead to a conclusion that an anamorphic *B. adusta* R59 is a promising strain for decolorization of a broad spectrum of polluted freshwaters and wastewaters. This fungus decolorizes lignin effluent and humic acids with different effectiveness, but in all cases a total visible decolorization of inoculated medium was finally observed. Therefore, the R59 strain may be recommended for bioremedation of polluted waters, although its properties should be optimized (by mutagenization methods) in order to increase its decolorizing effectiveness. Such treatment may result in shortened time of bioremediation performed by this fungus. Modification of *B. adusta* R59 strain will be a subject of our further research.

Acknowledgements

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