Use of central composite design of experiments in the delignification of olive oil processing waste pomace by Phanerochaete chrysosporium during solid state fermentation

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ABSTRACT

Ability of *Phanerochaete chrysosporium* to delignify the olive oil processing waste pomace (OWP) during solid state fermentation (SSF) was experimentally determined while the central composite design (CCD) technique, a response surface methodology (RSM), was used to study this bio-delignification process. A three level, three variable design was adopted. The pH of the culture system (x_1) (i.e., 4, 5 and 6), size of the inoculum (x_2) (i.e., 10, 20 and 30% v/w) and the frequency of aeration (x_3) (i.e., once every three days, once in every two days and once in every other day) were considered as independent variables. The influence of these three variables on the dependent variable, i.e., delignification of the OWP, was investigated. Response was represented mathematically by second-order quadratic equation and assessed using polynomial multiple regression model. Analysis of variance (ANOVA) showed a high coefficient of determination (R^2) value of 0.9448, thus ensuring a satisfactory adjustment of the regression model with the experimental data. Importance of the joint effects of the amount of the inoculum (x_2) and frequency of the aeration (x_3) and also interaction between pH (x_1) and the inoculum size (x_2), were discussed in terms of the delignification of the substrate during the SSF procss. When the aeration frequency was at the high level (i.e., one every other day) and the inoculum size was at the low level, the bio-delignification was at the maximal level.

KEYWORDS:

olive oil processing waste pomace, lignin degradation, Phaerochaete chrysosporium, central composite design, response surface methodology

1. INTRODUCTION

Olive oil is a typical and valuable agro-industrial product. Extraction of the oil from olive fruit generates large amount of organic wastes. The average annual global production of olive oil is about 1.6 million tons. The extraction of the oil is either by means of a batch press (classical process) or a solid/liquid centrifuge technique (centrifugal process). The olive oil manufacturing process usually yields an oily phase (20%), a solid residue 30% and an aqueous phase (50%) [1,2]. Degradation of the phenolic compounds responsible for the organic load and black color of the olive processing waste is the limiting step in the treatment of this waste, i.e., conventional

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biological processes for the treatment of these solid and liquid wastes are ineffective. Stringent environmental regulations impose increasing efforts toward the development of new technologies and improved methods for reduction of the bio-recalcitrant organics in wastes, such as those in the olive oil processing wastes. The problem of severe limitations which are introduced by these agro-industrial wastes is not only in the Mediterranean regions [3] but in other countries where olive is produced, similar situations can be found such as in the Middle-East countries. Use of white-rot fungi to degrade the phenolics in the olive processing wastes has been reported by several investigators [2, 4, 5].

In the case of *Phanerochaete chrysosporium* as the most studied fungus, the ability of the microbe to degrade these types of the refractory compounds are known to be due to the expression and activation of the lignin-degrading enzymatic system [6, 7, 8, 9]. Regarding lignin degradation, much focus has been on *P. chrysosporium* and this bio-event still, is poorly described at the enzymological level for other microorganisms. Production of ligninolytic enzymes, i.e., lignin peroxidase (LiP) and manganese dependent peroxidase (MnP) in *P. chrysosporuim* occurs during second phase of the growth under nitrogen or carbon limited environment and there are factors cause inactivations of these heme containing glycoproteins. LiP is easily over-oxidized by excess H_2O_2 and high levels of this co-substrate which is mainly produced indigenously, converts LiP to an inactive form. Loosely bound heme group to the LiP, can be lost under high-speed agitation [10]. Rapid fungus growth occurs under normally aerated conditions while the performance of the fungus in the enzyme production phase, was found to be considerably better under 100% oxygen atmosphere and it is pointed out that under oxygen-limited conditions thickness of the mycelia with the extracellular production of polysaccharide slime, have adverse effects on the enzyme formation and its activity [10]. Potential applications of the fungal peroxidases in treating bio-recalcitrant compounds in wastes of different industries have been discussed [9].

Solid-state fermentation (SSF) is defined as the growth of microorganism on solid materials not in liquid state and moist solid material may be stationary and the microorganism develops on the substrate surface similar to its growth in nature. Use of SSF on the industrial basis for the production of enzymes, chemical has been reported while SSF process also has been used for bioremediation and bidegradation of hazardous and toxic compounds [11].

Extracellular lignin degrading fungal enzymes act as a chemical drill for penetrating the substrate i.e., in SSF and for converting lignin to metabolic products and it is important to characterize the substrate for SSF process. Moisture content, particle size, specific weight and air volume are among major operative variables, for instance chopping and milling lignocellulosic materials is considered as a principal process step in a SSF and in this way, the surface to volume ratio of the substrate is increased providing better conditions for the microbial attack on the substrate. Although smaller size of substrate (<1mm) is not necessarily better for the conversion, since porosity of the substrate could be reduced and this may result in oxygen mass transfer limitation [12].

Different methods of physical, chemical and biological techniques have been used to break down the lignin in the agriculture residues and the depolymerization results in the releasing polysaccharide, sugars and phenolics [13]. Alkali-treated olive pomace is found effective pre-treatment for delignification of the pomace by several fungi, including *P. chrysosporium* [14]. In fact, swelling and structural disintegration of the cell wall increase susceptibility of substrate for the enzymatic attack of the microbe.

Experimental design considers simultaneously several factors and attempts to characterize the relationship between the independent variables and one or more dependent or response variables. Response surface methodology (RSM) is an effective tool for optimizing a process in which several factors and their interactions affect desired response [15]. RSM uses an experimental design such as the central composite design (CCD) to fit a model by least squares technique [16]. Adequacy of the proposed model is then revealed using the diagnostic checking tests provided by analysis of variance (ANOVA). The response surface plots can be employed to study the surfaces and locate the optimum. In recent years, use of RSM in the evaluation of biological processes has gained importance and is becoming an innovative approach in many research studies and industrial operations [17, 18]. Proper substrate, adequate amount of moisture, aeration, pH and temperature are among the culture conditions which affect significantly on the lignin degradation during SSF. Nutritional value of the olive oil is a fact and many countries are involved in the growing olivetrees program. In spite of great amount of works on the waste water treatment of olive oil agro-industry, study on the solid waste such as olive oil pomace is rather limited. The objective of the present work was to study removal of lignin from olive oil processing waste pomace (alkali-treated) by *P. chrysosporium* using solid-state fermentation process. A CCD in the form of a 2^3 full factorial design was used to develop a mathematical equation in terms of the lignin removal, providing quantitative evaluation of the SSF used to study bio-delignification process. pH, aeration and the inoculum size were controllable factors affecting the fungus performance in the process.

2. MATERIALS AND METHODS

2.1. Olive processing waste pomace (OWP)

The OWP used in this study was obtained from an olive oil mill in the Roodbar region, home of the olive growing and processing sector, in the northern part of Iran. The three phase centrifugation method has been used for the oil extraction. Fresh OWP was transported to our laboratory under refrigeration temperature within 15 hours of its production, and in order to carry out all the tests with the same sample, appropriate amounts of OWP were distributed in plastic bags and stored at –20°C until use. At the time of use, the OWP sample was thawed in a refrigerator, the samples then, dried in an oven at 75°C for 48 hrs. The sample was ground in a laboratory blender, and then a sieve with an appropriate size was used (the particle size of the substrate used was <1mm diameter). The OWP had the following composition (g per 100 g dry matter): moisture, 55; lignin 46.05; total phenolics 1.2.

In order to treat the substrate with NaOH, an appropriate amount of the dried ground pomace (200 g) was mixed with 3% w/v NaOH (300 ml) and the mixture held overnight at the room temperature.

To use the alkali-treated pomace for the SSF process, the moisture level of the substrate was set as 55% and this was done by adding sufficient volume of distilled water along adjusting pH at the predetermined value using 0.1N HCl. These adjustments were performed before sterilization. The substrate was sterilized in two consecutive days by autoclaving at 15 psi each time for 20 min.

2.2. Microorganism cultivation and preparation of the mat of mycelium

P. chrysosporium (5270) was purchased from the culture collection center of the Iranian Research Organization for Science and Technology. The fungus was grown on potato dextrose agar (PDA) and the plates were incubated at 25°C for one week. These plates were then stored at refrigerator until needed. At the time of use, these colonized plates were cut into small portions (~ 1 cm^2 cubes) using sterile cutter and the content of the one plate was transferred into a 250 ml Erlenmeyer flask having 100 ml potato dextrose broth. The flasks then incubated in a shaker incubator (60 rpm) at 25°C for one week. The formed mycelial mass was blended for 1 min in a sterile lab. blander. The homogenate obtained then was used to inoculate flasks of the alkali-treated OWP.

2.3. The SSF process

25 g of the alkali-treated pomace was placed into the 250 ml flask while the substrate having moisture content adjusted at 55%, and pH has been set at the predetermined level. The sterilized content of the flask was mixed with a selected level of the inoculum (%v/w). The flasks were incubated at 25 °C for 30 days. Aeration was through a tube connection installed in the flask cap, by introducing a flow of moist, sterile air while the duration of aeration was 15 min and frequency of the aeration was done, according to the design of the experiments used in the present study (Table 1).

Table 1. The level of regressor variables used in the present study[1].

pH (x_1)	inoculum size (% v/w, x_2)	frequency of the aeration* (x_3)
4 (-1)	10 (-1)	less frequent (once every three days) (-1)
5 (0)	20 (0)	middle level (once every two days) (0)
6 (1)	30 (1)	more frequent (once every other day) (1)

[1] The coded values for the variable are given in the parenthesis.
* less frequent: once every three days
middle level: once every two days
more frequent: once every other day

2.4. Analytical methods

The concentration of phenolics was determined using the Folin-Ciocalteau reagent after prior extraction of the OWP sample with ethyl acetate according to the protocol described [19, 4]- caeffeic acid was used as the standard. Lignin content was measured according to the method described in elsewhere [20]. Delignification was determined as:

$$\frac{(lignin\ content)_{t_0} - (lignin\ content)_{t_t}}{(lignin\ content)_{t_0}} \times 100$$

where t_0 and t_t were the amount of the lignin at the zero time and the time t during the process.

2.5. Experimental design and data analysis

As shown in Table 2 a CCD in the form of 2^3 full factorial design was used. The first eight treatment combinations form a 2^3 factorial design. The next six treatment combinations are referred to the axial runs, because they lie on the axes defined by the design variables. The last treatment combination represents the center run and this arrangement of CCD as shown in Table 2 is in such a way that allows the development of the appropriate empirical equations (i.e., second-order polynomial multiple regression equation) [15, 16].

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 \quad (1)$$
$$+\beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3$$

The predicted response (y) was therefore correlated to the set of regression coefficients (βs): the intercept (β_0), linear ($\beta_1, \beta_2, \beta_3$), interaction ($\beta_{12}, \beta_{13}, \beta_{23}, \beta_{123}$) and quadratic ($\beta_{11}, \beta_{22}, \beta_{33}$) coefficients. The "Design Expert" software was used for regression and graphical analyses of the data obtained in this experiment. The level of regressor variables used in this work are given in Table 1.

3. RESULTS AND DISCUSSION

3.1. Central composite design and fitted regression model as related to the bio-delignification process

In the present work, the relationship between the delignification ability of *P. chrysosporium* on olive oil pomace and three selected controllable factors namely pH, inoculum size and aeration frequency as related to the solid state fermentation, was studied. A CCD shown in Table 2 allows the development of mathematical equation where the response variable (delignification ability of the fungus on OWP) is assessed as a function of pH (x_1), inoculum size (x_2) and aeration frequency (x_3), and calculated as the sum of a constant, three first order effects (terms in x_1, x_2 and x_3) three interaction effects (terms in x_1x_2, x_1x_3 and x_2x_3) and three second-order effects (x_1^2,x_2^2 and x_3^2), according to the equation 1. The results obtained were then analyzed by ANOVA to assess the goodness of fit (i.e., significance test for the goodness of fit of theoretical data). Only the terms found statistically significant were included in the model. β_1, β_{11} and β_{22} coefficients were non-significant. Therefore, these coefficients were dropped from the model and a new ANOVA was then performed for the reduced model. This model was significant by the F-test at the 5% confidence level (prob>F<0.05). The following, fitted regression model (equation in terms of the coded values for the regressors) was used to quantitatively investigate the effects of pH, inoculum size and aeration frequency on the characterization of the delignification of the olive oil processing waste pomace by *P. chrysosporium*:

$$y=22.97+3.54x_2-2.58x_3-14.68x_1x_2+4.55x_1x_3-8.45x_2x_3+6.47x_3^2 \quad (2)$$

Table 2. Arrangement of the CCD for the three independent variables used in the present study. The response is also presented.

Variable levels/ coded values				Response (biodelignification) (%)	
Experiment no.	x_1	x_2	x_3		
1	-1	-1	-1	7.87	(9.885)
2	1	-1	-1	33.87	(30.149)
3	-1	1	-1	65.08	(63.233)
4	1	1	-1	26.40	(24.788)
5	-1	-1	1	13.14	(12.542)
6	1	-1	1	51.34	(50.986)
7	-1	1	1	30.56	(32.077)
8	1	1	1	16.03	(11.812)
9	-1	0	0	23.58	(22.965)
10	1	0	0	23.71	(22.965)
11	0	-1	0	20.54	(19.422)
12	0	1	0	24.12	(26.509)
13	0	0	-1	26.85	(32.014)
14	0	0	1	23.20	(26.854)
15	0	0	0	21.55	(22.965)
16	0	0	0	24.30	(22.965)

* Theoretically predicted value is given in paranthesis.

Statistical parameters obtained from the ANOVA for the reduced model of the bio-delignification are given in Table 3. Since R^2 always decreases when a regressor variable is dropped from a regression model, the adjusted R^2 which takes the number of regressor variable into account, is usually selected in statistical modeling [16]. The R^2 coefficient gives the proportion of the total variation in the response variable explained or accounted for by the predictors (xs) included in the model. The R^2 coefficient in the present study ensured a satisfactory adjustment of the regression model to the experimental data. The predicted sum of squares (PRESS) which is a measure how a particular statistical model fits each point in the design is 503.77. The adequate precision value, which is an indication of the ratio of the 'signal to noise' was found to be 24.67 which is indicative of an adequate signal. A ratio of more than 4 is said to be desirable.

Table 3. Statistical parameters obtained from the analysis of variance for the reduced model fitted for the bio-delignification of olive oil pomace.

Variable	Biodelignification
R^2	0.9669
R^2 adjusted	0.9448
F-value	43.8
Prob>F	<0.0001
Std.Dev.	3.27
Coefficient of variance	12.11
PRESS	503.77
Adequate precision	24.67

Fig. 1 shows the coefficients of the regressors included in the polynomial equation 2 and it can be seen that the interactions due to the pH-inoculum size (x_1x_2) and inoculum size -aeration frequency (x_2x_3) were important. Table 2 shows the experimental and the predicted values for the biodelignification. Treating agricultural residues and agro-industrial wastes by applying SSF process using ligninolytic microbes (i.e., white-rot fungi) is an attractive approach since the digestibility and feed value of the converted substrate can be considerably improved [21, 22, 11].

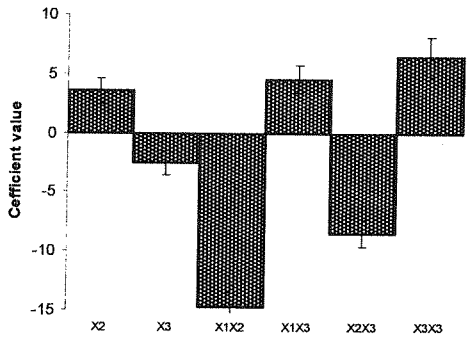

Fig.1. Coefficient of the regressors in the fitted polynomial equation used in the present study (Eq. 2).

In the present work, pH, inoculum size and aeration frequency were selected although still, there are many other factors which critically influence this type of the SSF process. The contribution of each of these three factors and their interactions to the delignification variable (y) was directly measured by the respective regressor coefficient in the fitted model. A positive sign for the coefficient β_2 in the fitted model indicated that the level of the delignification increased with increased levels of the factor x_2 namely, the amount of the inoculum. The negative effect of the aeration frequency (x_3) and of the interaction between the inoculum size and aeration frequency (x_2x_3) on the bio-delignification (y), indicated that a simultaneous increase in the fungal load with a decrease in the aeration frequency, leads to an increase in the response. Also the ability of the fungus to delignify the OWP increases as the interaction of the pH and the size of the inoculum decreases, as it is seen for the coefficient of x_1x_2. In order to gain a better understanding of these results, the predicted models are presented in Fig. 2 as the three dimensional response surface plots.

3.2. Response surface plotting and optimization of the bio-delignication of the OWP

For the graphical interpretation of the interactions between the regressors in the polynomial equation, the use of the 3-D plots of the fitted regression model is highly recommended [15, 16]. Regressor variables giving interaction terms with the largest absolute value for the coefficients in the fitted model were chosen for the axes of the response surface plots.

Fig. 2a shows the calculated dependence of the delignification of OWP on the dimensionless amount of inoculum x_2 and on the dimensionless aeration frequency x_3 for the SSF process conducting at pH 5 (x_1=0). When the amount of the inoculum is low, the delignification during the SSF is smallest at the low level of the aeration frequency (once every three days) and the delignification increases as the frequency of the aeration increases (once in every other day). As the size of inoculum increased (x_2) and reached a constant level at the central level of the aeration frequency (x_3), the increasing trend of the delignification is stopped, thereafter, as the fungal load increases (x_2) the response variable decreases with the aeration becomes less frequent (once every three days) (see Fig. 2a).At constant level of variable x_1 (pH 6), the delignification is increased with increasing the aeration frequency (x_3) at low level of the inoculum size (x_2) while y is reduced with increasing the frequency of the aeration (once every other day) at high level of the fungal load (Fig. 2b).

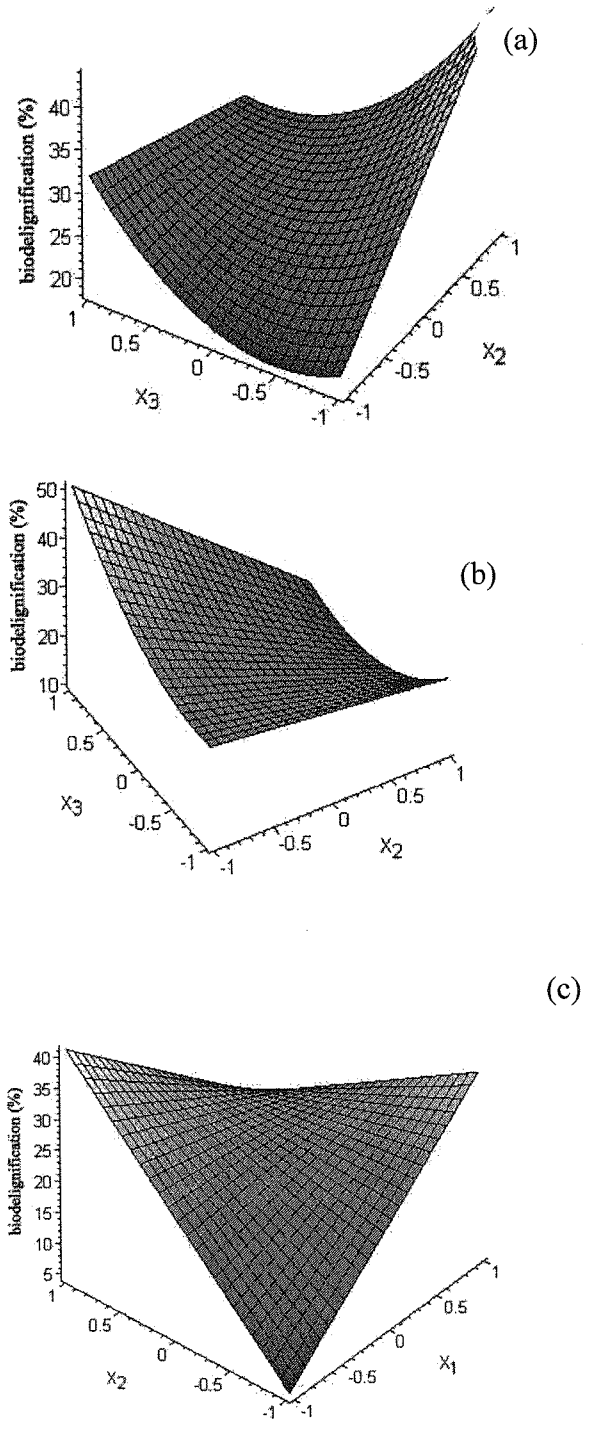

Fig. 2. Second-order response surface plot in the bio-delignification of olive oil pomace(y).

Dependence of y on the inoculum size (x_2) and aeration frequency (x_3) (pH, x_1=0) (a).

Dependence of y on the inoculum size (x_2) and aeration frequency(x_3) (pH$_1$ x_1=1) (b).

Dependence of y on the pH(x_1) and the inoculum size (x_2) (aeration frequency, x_3=0) (c).

The effect of oxygen and manganese on LiP and MnP activities using shallow stationary cultures of *P. chrysosporium* in nitrogen-limited cultures was studied and in those cultures without Mn(II), significant levels of LiP activity were observed under normal aeration but when Mn was added to the media at level of > 13 mg/*l*, no LiP was detected (suppressive effect of Mn on LiP formatation) [23]. On the other hand, detecting high levels of LiP activities in the cultures containing Mn and in the presence of oxygen was found to be related to the formation of high levels of MnO_2 species which act efficiently as H_2O_2 scavenger, and in this cellular manner LiP can be protected from inactivation by H_2O_2 produced intracellularly [1]. The results of the present study show importance of the inoculum size and the frequency of the aeration (see Figs. 2a and b).

The basic principles of the two fungal processes are not contrary to each other (the mycelial growth and the ligninolytic enzyme production) [10]. The enzymes activities were not measured in the present study but the results obtained, showed that lower frequency of the aeration led to less mycelial growth and higher biodelignification, probably these were due to higher levels of the ligninolytic enzymes. As Leisola and Waldner, [10] pointed out, higher aeration and thicker mycelial growth eventually create lower oxygen availability to the culture and this results in lower levels of the ligninolytic enzymes production (i.e., less biodelignification).

Fig. 2c shows the predicted influence of the non-dimensional pH(x_1) and the non-dimensional amount of the inoculum (x_2) on the delignification of the OWP when aeration frequency was set at the central level (x_3=0). At the low level of pH (i.e., pH 4), the delignification increases with increasing inoculum size. The delignification was at lowest level when two of the regressors, namely pH and inoculum size were both at the low levels (Fig. 2c).

Capacity of *P. chrysosporium* to delignify a particular substrate is under influence of the culture pH. Perez and Jeffries [1] studied the effects of buffer and pHs on the ligninolytic enzymes expressed by *P. chrysosporium*. Effect of the pH shift during growth of the culture on the LiP repression was tested. At the high pH no repression was detected and in the presence of the high levels of Mn(II), production of the LiP delayed (formation of the Mn(II)- Mn(III) complex in the culture). Following formation of MnO_2 and its precipitation, the LiP activity was detected again [1]. Is addition, the MnP activity at pH 5 was higher (3 times) than at pH 4.5 [1]. In the present study, the increased level of the biodelignification at pH 4 and at the low frequency of the aeration (once every three days) could be an indication of the LiP activity. Further during the growth, low oxygen availability created by the mycelial growth may repress the LiP activity. On the other hand and at the high pH level, the MnP activity may be operative in the bio-delignification process. The LiP and MnP enzymes both have critical role in this bio-treatment

and more works are needed to obtain a clear picture about the role(s) of these enzymes in the olive oil pomace bio-treatment.

4. CONCLUSION

As it is stated in literature delignification of agricultural residues by *P. chrysosporium* is an efficient way for treating these types of wastes which is first step in converting them to a valuable product such as animal feed. Many factors affect the performance of the fungus. The bio-delignification was highest when two of the regressors namely, pH and inoculum size were at low and high level, respectively. By considering the aeration frequency at the high level (once every other day) and the size of the inoculum at low level, the bio-delignification was at the maximal level. Results of the present work show that use of a statistical design of the experiment is a helpful approach in defining the delignification of the substrate more properly. Cellular activities such as ligninolytic enzymes activities, are under influence of the environmental factors. Measuring these enzymes activities during the biodelignification process could give better position to the design of the experiment in terms of the interpretation of results.

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