Identification of markers at various stages of batch fermentation and improved production of xylanase using *Aspergillus niger* (KP874102.1)

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Received: 16 January 2018 / Accepted: 18 July 2018 / Published online: 24 July 2018
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Abstract

Improved xylanase production was carried out through optimization of environmental stresses during spore preservation, seed cultivation and batch fermentation and identifies the markers at various stages. The maximum spore size (radius 6.5 µm) of *Aspergillus niger* was noticed after 28 days of spore preservation. During seed cultivation, the hypha formed alongside of germination tube (length 196.8 µm) was noticed only at pH-7 after 18 h of incubation at 28 °C. Therefore, pH-7 and 28 °C were considered as optimum during seed cultivation. In this stage, the final pH of the medium was found to be 6.2 which can be used as marker for completion of seed culture. The production media was optimized through Taguchi methodology. The maximum xylanase production was found to be 1575.93 U. The optimum concentration for media components was found to be xylan from beechwood of 3 g/l, potassium nitrate of 10 g/l, magnesium sulphate of 5 g/l, di-potassium hydrogen phosphate of 50 mM, calcium carbonate of 2 g/l, 1000× of trace element (1 ml) and sodium chloride of 5 g/l. It is evident that improved production of xylanase can be possible through optimization of environmental stresses during spore preservation, seed cultivation and batch fermentation and can be intensified through identification of markers at various stages of fermentation process.

Keywords Bioproducts · Fermentation · Enzymes · Fungi · Optimization

Introduction

The xylanase enzymes are produced by a wide range of microorganisms including bacteria and fungi, plant and animal cell (Ahmed et al. 2009; Knob and Terrasan 2010; Uday et al. 2016c). They are either extracellular or intracellular in nature. Since the plant and animal xylanase are unable to fulfill the current world demand, there is an increased interest

for microbial xylanase. The fungus of the genus *Aspergillus* is widely used for the production of commercial extracellular xylanase. Xylanase has wide applications in industries such as production of biofuel, bulk chemicals, enzymatic treatment of animal feeds to release pentose sugars, bio-bleaching of wood pulps, food additives in baking industry, ingredients in laundry detergents and fabric care compositions (Ahmed et al. 2009; Knob and Terrasan 2010).

Keeping in view of the demand, usefulness and cost effectiveness of the xylanase, it is very important to focus on their commercial production through submerged fermentation. It is obviously true that process parameters like optimum media composition and physiochemical parameters play a vital role in the improvement of microbial growth as well as xylanase production. Since microbial xylanase production is a complex process, optimization of process parameters is an important task for biochemical engineers with regard to its improved yield (Uday et al. 2016a, b). In addition, morphology of filamentous fungi has a significant role on the rheological properties

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of cultivation medium which indirectly affects microbial production of secondary metabolite. Optimization of seed cultivation intended for cost effective fermentation process is a primary task for biochemical engineers. The quality of seed culture depends on spore age along with nutritional and physicochemical parameters (Dantigny and Nanguy 2009). Additionally, media components as well as environmental factors influence the behavior and the metabolic activity of the spores. As far as our knowledge is concerned, most of the research works focus on fungal growth, germination and control of morphology in fungal xylanase fermentation. However, limited research has been focused on physiological and biochemical aspects of fungus spores age along with preservation time for seed culture development. In fermentation, identification of markers for completion of seed cultivation is also utmost important for bioprocess engineers. Therefore, the potential significance of these parameters was forcefully brought to our attention on xylanase production by an experiment with *Aspergillus niger* (KP874102.1). In addition, control of morphology of fungi is prerequisite as it strongly influences the physical property of fermentation broth during seed culture.

Therefore, the present work was focused on process intensification through optimization process factors during spore preservation, seed cultivation and batch fermentation using newly isolated *Aspergillus niger* (KP874102.1). In addition, marker identification for completion of seed cultivation was also critically evaluated.

Materials and methods

Chemicals and analysis

Beech wood xylan was purchased from Sigma (Sigma Aldrich Co. Ltd., USA). DNS reagent (Merck India), sodium potassium tartrate (SRL, India), sodium hydroxide (SRL, India), sodium sulphite (Merck, India), tri sodium citrate (SRL, India), potato dextrose agar (SRL, India), magnesium sulphate (Merck, India), ferric chloride (SRL, India), copper sulphate (Merck, India), bovine serum albumin (Himedia, India), xylose (Sigma, USA), and bradford reagent (Himedia, India) were used in this study. All other chemicals (of analytical grade) used, are commercially available in India. The statistical software package, Qualitek-4 software (Nutek Inc., MI, USA) was used to analyze the experimental design and for the analysis of the experimental data. Optical Inverted Microscope (Made-Olympus, Model No: CKX41) with Mshot Digital Imaging System 9.0 (Microshot Technology Co. LTD) was used for microscopic analysis. PC Controlled Double Beam UV–VIS Spectrophotometer (Thermo Fisher Scientific, USA) was used for analytical purpose.

Microorganism

Xylanase producing strain, *Aspergillus niger* (KP874102.1), was isolated from soil sample (Barmuda forest, Tripura west, India) and characterized. The above isolated strain was grown on PDA media for 5 days at 28 °C. After incubation the slants were stored at 4 °C for further experiment.

Optimization spore age and preservation time

In this study, the spore of the slant at different preservation time (7, 14, 28, 42 and 56 days) was suspended with sterile distilled water and observed under Optical Inverted Microscope (Made-Olympus, Model No: CKX41).

Seed culture

Erlenmeyer flask (250 ml) containing 50 ml medium was inoculated with 10^7 spores per ml which was incubated at 28 °C in a rotary shaker at 120 rpm. Inoculum was prepared by suspending the spores from a PDA slant by adding sterile distilled water. The spore suspension was then filtered and again suspended in sterile deionized water to give a final spore count of 1×10^7 spores/ml which was counted using haemocytometer (Anderson and Smith 1972). The seed medium used was composed of (g/l, w/v) xylan, 1; KNO₃, 10; MgSO₄·7H₂O, 5; NaCl, 5 and a 1000× trace element solution; 1 ml/l (pH 7) trace element solution contains (g/l, w/v) ZnSO₄·7H₂O, 20; H₃BO₃·10; MnCl₂·7H₂O, 5; FeSO₄·7H₂O, 5; CoCl₂·5H₂O, 1.5; CuSO₄·5H₂O, 1.5; Na₂MoO₄·4H₂O, 1) (Uday et al. 2016a).

Optimization of seed culture

In the optimization study, pH and temperature were only considered as important factors on germination of spore during seed culture. The spore having optimized age was incubated at different combinations of pH and temperature. The pHs selected for this study were 5, 5.5, 6, 6.5, 7 and 7.5. The effect of germination of spore was carried out at temperature of 25, 28, 30, 35 and 40 °C at optimized pH. The aliquots of samples were taken at 3-h intervals of time and the length of germination tube observed under Optical Inverted Microscope (Made-Olympus, Model No: CKX41).

Optimization of production media

The production media was optimized through Taguchi methodology (1986). In this study, carbon source, nitrogen source, metal ion source, sodium chloride, K₂HPO₄ and CaCO₃ were considered as important factors for xylanase

production for *Aspergillus niger* (KP874102.1). All the variables were investigated at three widely spaced levels shown in Table 1. The levels of factor were decided from previous unreported work. Selection of most suitable carbon, nitrogen and metal ion was done by “change one variable at a time (COVT)” method. Xylan from beechwood, potassium nitrate and magnesium sulphate were found to be the higher yielding carbon, nitrogen and metal ion source, respectively, for xylanase production.

The experiments were carried out at 28 °C in a rotary shaker (Benchtop Incubator Shaker, Scigenics Biotech, India) at 120 rpm for 7 days in a shake flask (250 ml) containing 50 ml working volume. A 5% of seed culture was inoculated in production media, containing (g/l, w/v) of xylan (2, 3 and 4); KNO₃ (7.5, 10 and 12.5), NaCl (2.5, 5 and 7.5), magnesium sulphate (2.5, 5 and 7.5), K₂HPO₄ (2.5, 5 and 7.5), and CaCO₃ (2, 4 and 6) and 1000× of trace element (1 ml, 2 ml and 3 ml). The pH of the medium was maintained at 7. In this study, three levels of seven factors

(Tables 1, 2) were considered for xylanase production and the size of experimentation was represented by symbolic array of L-18 (which indicated 18 experimental trials). The culture was centrifuged using refrigerated table top centrifuge (Sigma; 2-16KL, Germany) at 15,000×g for 10 min at 4 °C and the supernatant was used for assay of xylanase, residual xylan and reducing sugar.

Xylanase assay and protein measurement

Xylanase activity was determined according to modified Baileys et al. (1992) and reducing sugar was measured using the 3,5-dinitrosalicylic acid. Briefly, 200 µl of fermentation supernatant was added to the reaction mixture that contained 1.8 ml of 1% (w/v) beechwood xylan dissolved in 0.2 M Potassium Chloride buffer (pH 2.0) and kept at 50 °C for 10 min. The amount of reducing sugar liberated was determined by the 3,5-dinitrosalicylic acid (DNS) method using xylose as a standard (Miller 1959; Uday et al. 2017).

Table 1 Experimental range of the seven numerical variables studied using Taguchi methodology

Serial no.	Coded factor	Factor	Level-1	Level-2	Level-3
1	A	Xylan from beechwood (g/l)	2	3	4
2	B	Potassium nitrate (g/l)	7.5	10	12.5
3	C	Sodium chloride (g/l)	2.5	5	7.5
4	D	Magnesium sulphate (g/l)	2.5	5	7.5
5	E	Di-potassium hydrogen phosphate (mM)	25	50	75
6	F	Calcium carbonate (g/l)	2	4	6
7	G	Trace element (ml)	0.5	1	1.5

Table 2 L₁₈ OA (3⁷) of design experiments for seven variables with actual xylanase production

Trial no.	A	B	C	D	E	F	G	Xylanase activity (U)		
1	1	1	1	1	1	1	1	343.75	308.33	377.08
2	1	2	2	2	2	2	2	800	720.83	879.17
3	1	3	3	3	3	3	3	285.42	314.58	256.25
4	2	1	1	2	2	3	3	856.25	943.75	770.83
5	2	2	2	3	3	1	1	739.58	822.92	904.17
6	2	3	3	1	1	2	2	685.42	754.17	616.67
7	3	1	2	1	3	2	3	502.08	410.42	456.25
8	3	2	3	2	1	3	1	637.5	779.17	708.33
9	3	3	1	3	2	1	2	770.83	943.75	856.25
10	1	1	3	3	2	2	1	352.08	320.83	287.5
11	1	2	1	1	3	3	2	439.58	400	360.42
12	1	3	2	2	1	1	3	570.83	514.58	629.17
13	2	1	2	3	1	3	2	629.17	566.67	691.67
14	2	2	3	1	2	1	3	1068.75	970.83	875
15	2	3	1	2	3	2	1	754.17	616.67	685.42
16	3	1	3	2	3	1	2	816.67	668.75	743.75
17	3	2	1	3	1	2	3	800	720.83	879.17
18	3	3	2	1	2	3	1	691.67	629.17	566.67

One unit of xylanase activity was defined as the amount of enzyme that catalyzes the release of 1 μ mol of xylose equivalent in one minute under the standard assay conditions. Protein concentration was determined by the method of Bradford using bovine serum albumin (BSA) as the standard (Bradford 1976). All experiments were done in triplicate.

Results

Optimization of spore age and preservation time

The physiological changes of spore with different preservation age (7, 14, 28, 42 and 56 days) are shown in Fig. 1. From the results (Fig. 1), it was noticed that the radius of spores increases with the preservation time upto 28 days. However, no significant morphological changes of radius were noticed after 28–42 days. However, significant decrease of spore radius was noticed after 56 days of preservation of spore. It is interesting to note that 2.5-fold enhancement of spore size (6.5 μ m) was observed in comparison with initial spore size after 28 days of preservation.

Optimization of seed culture

To evaluate the effect of pH on germination, the spore was inoculated at seed media (28 °C) at different pH viz. 5, 5.5, 6, 6.5, 7 and 7.5. The effects of pH on germination of spore during seed culture were reported in Table 3. Table 3 indicates that germ tubes were formed from most of the spores in the range of pH from 6 to 7.5 after 3 h of incubation. However, germination tube was noticed by 6 and 9 h of

incubation at pH of 5.5 and 5, respectively. The lengths of germination tube at 3, 6, 9, 12, 15, 18 h are illustrated at Fig. 2a–f, respectively. It is interesting to note that the hypha alongside of germination tube (Fig. 2f) was observed only at pH of 7 after 18 h of incubation at seed media and length was found to be 196.8 μ m. It is evident that the initial pH of seed medium was slowly decreased from 7 to 6.2 after 18 h of incubation.

To evaluate the effect of temperature on germination, the spore was inoculated to seed media (pH-7) at different temperature viz. 25, 28, 30, 35 and 40 °C. Results showed that different length of germination tube was formed after 3 h of incubation at seed media for all experiment temperature apart from 40 °C (Table 3). However, a complete inhibition of germ tube formation was found up to 9 h incubation at 40 °C. It was found that the same type of germ tube elongation was found at all temperatures. In addition, the length of elongation increased significantly with the incubation time. The optimum temperature for germination was found to be 28 °C at seed culture. However, hypha alongside of germination tube was noticed at 28 and 30 °C with germination tube length of 196.8 and 187.9 μ m, respectively, after 18 h of incubation at seed media.

Optimization of production media

The production media was optimized using Taguchi methodology and the experimental data were processed using Qualitek-4 software (Nutek Inc., MI, USA) to evaluate the influence of individual factors, multiple interactions of the selected factors and determination of optimum conditions for xylanase fermentation. Analysis of variance (ANOVA)

Fig. 1 The effect of radius of spore during spore germination on spore preservation time (**a** 7 days; **b** 14 days; **c** 28 days; **d** 42 days; **e** 56 days)

Table 3 The effect of pH and temperature on germination tube length

pH	3 h	6 h	9 h	12 h	15 h	18 h	Final pH
Length of germination tube (μm)							
5	–	–	16.3	20.6	23.7	27.9	4.5
5.5	–	15.2	18.8	24.9	27.3	32.1	4.5
6	21.7	28.5	38.3	45.8	51.2	55.1	4.6
6.5	76.7	82.1	101.2	130.2	143	160.3	5.5
7	94.4	102.4	130.9	173.8	188.8	196.8 ^a	6.2
7.5	90.3	99.7	127.1	164.1	176.5	186.3	6.5
Temperature (°C)	3 h	6 h	9 h	12 h	15 h	18 h	Final pH
Length of germination tube (μm)							
25	80.4	88.2	122.5	147.2	167.5	186.1	6.5
28	94.4	102.4	130.9	173.8	188.8	196.8 ^a	6.2
30	85.2	95.6	125.1	155.8	170	187.9 ^a	6.5
35	42.60	45.60	59.50	77.90	85.0	87.90	6.5
40	–	–	19.80	24.9	27.30	32.30	6.8

^aHyphae formed alongside of germination tube

was used to determine the variation of result due to each factor by analyzing through experimental data. The percentage of contribution of each factor with their interactions is reported in Table 4. Results showed that xylan from beechwood xylan contributed the maximum impact (50.02%) on xylanase production followed by potassium nitrate (13.56%), magnesium sulphate (11.32%), di-potassium hydrogen phosphate (9.19%), calcium carbonate (5.46%), trace element (4.18%) and sodium chloride (1.58%). Optimum condition of the factors and their performance with respect to their contribution to achieve high xylanase yield were shown in Table 5. Results showed that xylan from beechwood showed the maximum role on xylanase production followed by potassium nitrate, magnesium sulphate, di-potassium hydrogen phosphate, calcium carbonate, trace element and sodium chloride. Taguchi DOE design suggested that higher levels of xylanase yield can be achieved with xylan from beechwood (3 g/l), potassium nitrate (10 g/l), magnesium sulphate (5 g/l), di-potassium hydrogen phosphate (50 mM), calcium carbonate (2 g/l), 1000× of trace element (1 ml) and sodium chloride (5 g/l). The expected xylanase production at optimum condition in *S/N* ratio was found to be 64.221 (total contribution from all the factors being found 8.64 with grand average performance of 55.56). The estimated xylanase production from the *S/N* ratio was 1625.74 U with MSD of 0.000000378355 [by Eq. (3)].

Figure 3 illustrates the frequency distribution of current condition along with improved condition. It was evident from the Fig. 3 that the xylanase yield could be increased from 599.51 to 1625.74 U. The overall 171.17% enhancement in the xylanase yield could be achieved. Further to validate the proposed experimental methodology, fermentation

experiments were performed by employing the obtained optimized culture conditions. The experimental data showed that enhanced xylanase production (162.87%) was found with the optimized culture conditions.

Discussion

Optimization of seed cultivation is an important parameter for cost effective fermentative process. Since media components along with physicochemical factors influence the behavior and metabolic activity of the spores, the quality of seed culture depends on age of spore as well as nutritional and physicochemical parameters (Dantigny and Nanguy 2009). It has been found that most of the research was focussed on fungal growth and their germination. However, limited research has been focused on optimization of fungus spore age and preservation time for seed culture development. Therefore, the potential significance of spore age as well as preservation time was forcefully brought to our attention for seed culture development by an experiment with *Aspergillus niger* (KP874102.1). In the present study, it was found that the sporulation of *Aspergillus niger* (KP874102.1) was initiated after 2 days of inoculation and was completed within about 72 h. The spore age was counted as the age of the culture from where the spore was obtained. Preservation time of spore at 4 °C was measured after spore used for seed culture to spore was formed. The maturation of spore was determined by observing the black color on the plate. Figure 1 depicts that the increase of radius of spores is highly dependent on preservation time upto 28 days. In contrast, there are no significant morphological changes

Fig. 2 The effects of pH and temperature on germination tube length in seed culture after various time (**a** 3 h; **b** 6 h; **c** 9 h; **d** 12 h; **e** 15 h; **f** 18 h)

Table 4 Analysis of variance (ANOVA)

Factor	Xylanase production				Percent	P value
	SS	Variance	F ratio	Pure sum		
Xylan from beechwood (g/l)	90.24	45.12	91.82	89.26	50.03	<0.00001
Potassium nitrate (g/l)	25.17	12.59	25.61	24.19	13.56	<0.00001
Sodium chloride (g/l)	3.80	1.90	3.87	2.82	1.58	0.041202
Magnesium sulphate (g/l)	21.17	10.59	21.54	20.19	11.32	0.000022
Di-potassium hydrogen phosphate (mM)	17.38	8.69	17.69	16.40	9.19	0.00007
Calcium carbonate (g/l)	10.73	5.36	10.91	9.74	5.46	0.000895
Trace element (ml)	8.45	4.22	8.60	7.46	4.18	0.002628
Other/error	1.47	0.49			4.69	
Total	178.41				100%	

Table 5 Optimum condition for xylanase production

Coded factor	Factor	Values	Level	Contribution for xylanase production from S/N ratio
A	Xylan from beechwood (g/l)	3	2	2.04
B	Potassium nitrate (g/l)	10	2	1.56
C	Sodium chloride (g/l)	5	2	0.46
D	Magnesium sulphate (g/l)	5	2	1.52
E	Di-potassium hydrogen phosphate (mM)	50	2	1.22
F	Calcium carbonate (g/l)	2	1	1.02
G	Trace element (ml)	1	2	0.84
Total contribution from all factors				8.664
Current grand average performance				55.556
Expected result at optimum condition				64.221

Fig. 3 Performance distribution of current and improved condition for xylanase production

observed after 28–42 days. It was early reported that the spores were resistant to a variety of environmental stresses such as preservation due to hydrophobic nature as they have

thick walls, comprising a melanin and hydrophobic/rodlet layer (Dague et al. 2008). Since spore contains an abundance (~200 to 700 μ mol/g dry wt) of nutrient reserves

mainly D-trehalose and D-mannitol, they exhibit a low rate of metabolic activity, e.g. low rates of respiration, protein and nucleic acid synthesis during preservation having low water activity (Marleau et al. 2011). Therefore, 2.5-fold enhancement of spore size (6.5 µm) in comparison with initial spore size was noticed after 28 days of preservation. The significant decrease of spore size after 56 days is due to rapid consumption of reserve and water activity (Dantigny and Nanguy 2009). Therefore, the great differences in longevity of spores indicate the importance of judicious selection of culture media for maintenance of stock cultures.

The spores were commonly thought of as ‘resting cells’ and germinate when environmental conditions were favorable to support growth and development (Osharov and May 2001). Since spore size is greater after 28 days of preservation, spore age after 28 days of preservation was considered for seed culture. The spore germination in seed culture is a crucial event in the propagation of the species and, therefore, the factors that control spore germination of fungi were considered worth serious attention in this study. The extent of germination of most spores is influenced by media components and environmental factors such as temperature, pH, relative humidity, etc. (Sakthiselvan et al. 2014; Colin et al. 2013). In the present work, the experiment was carried out with optimized seed media which was developed in the laboratory and the effect of pH and temperature on germination of spore with respect to time was critically evaluated.

The time of germ tube formation as well as length of it was affected by the initial pH and temperature of seed culture (Table 3). Results showed that pH-7 and 28 °C were found to be optimum since higher length of germination tube was found and other pHs and temperature seemingly have less influence on length of germination tube when compared. It has been found that the initial pH was slowly decreased from 7 to 6.2 after 18 h of incubation at 28 °C in seed medium which is due to the production of organic acid when sugars are fermented (Sae-lee 2011). Therefore pH 6.2 can be used as marker for completion of seed culture.

The production media was optimized using Taguchi methodology (TM) since the application of TM has been quite common to optimize the process variables in biochemical process (Basak et al. 2013). This method of the studies involves for a given system a set of independent variables both controllable and uncontrollable over a specific region of interest (Kumar et al. 2013). It is also important that small-scale experiments are valid over entire experimental region; further ANOVA can be used to analyze the experimental data to find out the statistically significant outcome at optimum levels. The signal-to-noise ratio (S/N) analysis with bigger-is-better performance characteristics was used for all the experimental trials. In this methodology, the signal and noise represent the desirable value (mean) and standard deviation (SD),

respectively, for the output characteristic. The signal-to-noise (S/N) ratio is a performance measurement of a dynamic system to evaluate the robustness of the overall process (Zhou et al. 2010). The S/N ratio was employed to measure the qualification characteristic deviating from the desired value. A loss function [$L(y)$] was developed for the deviation (Mitra 1998) which was represented by $L(y) = k \times (y - m)^2$, where k , m and y denote the proportionality constant, the target value and experimental value obtained for each trial, respectively. The loss function can be written for bigger and better quality characteristics as $L(y) = k \times (1/y^2)$ and the expected loss function can be represented by

$$E[L(y)] = kE(1/y^2), \quad (1)$$

where $E(1/y^2)$ can be estimated from a sample of n as

$$\sum_{i=1}^n [1/y_i^2]/n. \quad (2)$$

The mathematical expression for the S/N ratio for the “bigger is better” case for the performance statistics that measures deviation from the target was called as mean square deviation (MSD) and given by:

$$Z = -10 \log(\text{MSD}) = -10 \log \sum_{i=1}^n [1/y_i^2]/n. \quad (3)$$

In conclusion, it may be stated that the present investigation reports an in-depth study of the effects of environmental stress during spore preservation and seed cultivation using newly isolated *Aspergillus niger* (KP874102.1). The evaluation of the effects has been carried out based on morphology. Temperature and pH control strategies were applied for improved quality of seed culture. In the present study, optimum spore preservation is found to be 28 days. Within the range of experimental study the optimum temperature and pH are found to be 28 °C and 7, respectively, during seed cultivation. It is interesting to note that the approximately equal length of germination tube was noticed after 36 h of seed culture when fresh spore was inoculated at same seed medium (data not shown). However, in present study the seed cultivation process is completed within 18 h through optimization of spore preservation time along with optimization of temperature and pH of seed cultivation. Therefore, the present seed cultivation process would be intensified because of lower operating cost in comparison to the previous process. In the present investigation, pH (6.2) is considered as marker for completion of seed cultivation. In the batch study, the production media was optimized using Taguchi methodology and significant enhanced xylanase production (1625.74 U) is found after 7 days of fermentation.

Funding This material is based upon work supported by the National Institute of Technology, Agartala, India. The authors would like to acknowledge the National Institute of Technology, Agartala, Ministry of Human Resource and Development, Government of India, for Fellowship (0000-0003-4637-991X).

Compliance with ethical standards

Ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflicts of interest.

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