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Deodar (*Cedrus deodara*) wood dust: An alternative substrate for amylase production in solid state fermentation by alkalophilic *Bacillus* spp. A1 isolated from mushroom compost

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ABSTRACT

Different agro-residues were evaluated as substrate in solid state fermentation for amylase production by alkalophilic *Bacillus* spp. A1 isolated from mushroom compost. Various fermentation parameters were optimized for enhanced amylase production under solid state fermentation (SSF). The organism was able to utilize all the lignocellulosic substrate giving maximum amylase activity (264.4 ug^{-1}) on deodar wood dust followed by wheat bran (233.0 ug^{-1}) and maize cob (168.5 ug^{-1}) at 48h of incubation period. Maximum amylase activity was found at pH 10.0 and temperature 35^{0}C. The production of the enzyme activity was found to be growth associated as maximum amylase activity corresponded to maximum viable count irrespective of the substrate used. The crude amylase preparation from alkalophilic *Bacillus* spp. A1 was partially characterized. Maximum amylase activity 288.2Ug^{-1} DBD was observed when 1.5 per cent starch was used as substrate for amylase activity. The enzyme was stable up to 55^{0}C at pH 10.0 for 15 minutes. The study concludes that *Bacillus* spp. A1 under solid state fermentation using cheap and annually renewable substrate *i.e.* deodar wood dust can reduce the cost of enzyme production.

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Key Words : Amylase, Solid state fermentation (SSF), *Bacillus* sp., Deodar wood dust

INTRODUCTION

Among various microbial enzymes, the introduction of amylases for commercial purpose represents a milestone in the industries and are of great significance in present day biotechnology. Microbial amylases are very diverse in their enzymatic properties, behavior and substrate specificity which make them attractive for many industrial applications like starch processing, milling, brewing, paper, textile, food and feed industry (Pandey *et al.,* 1999). Two major classes of amylases have been identified in micro-organisms, namely alpha-amylase and gluco-amylase. Alpha-amylase (endo-1.4,α-D-glucon,EC 3.2.1.1) are extracellular enzymes that randomly cleaves the 1,4-α-D-glucose units in the linear amylase chain. Glucoamylase (exo-1,4-α-D-glucan glucanohydrolase, EC 3.2.1.3) hydrolyzes single glucose units from the non-reducing ends of amylase and amylopectin in a step wise manner (Pandey *et al*., 2000a). Most of the enzymes reported for mesophilic bacteria have activity optima in the acidic to almost neutral pH range. A few alkalophilic *Bacillus* spp. have their pH optima between 8.0 to 9.5 and display maximum catalytic activity at 40^0, 55^0 and 60^{0}C. Alkaline amylase is found to be more important commercially. The main areas where use of alkaline amylases has expanded are household laundary, automatic dish washers, and industrial and institutional cleaning (Hagihara *et al.,* 2001). To ensure the commercial utilization of hemicellulose residues, the production of large quantities of amylase at low cost will be required. The enzyme production by solid state fermentation (SSF) is gaining importance due to its potential advantages in production of enzyme in high yield at high concentration (Mamo and Gessesse,1999; Pandey *et al.,* 2001; Ramdas *et al.,*1996). Solid state fermentation process is significantly influenced by the nature of solid substrate. In this respect, the utilization of annually renewable agro industrial wastes as a substrate represents an essential step to reduce the cost of enzyme production. However, bacterial strains belonging to genus *Bacillus* are reported

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to produce extracellular enzymes in SSF (Shankaranand *et al.,* 1992; Ramesh and Lonsane, 1987; Krishna and Chandrasekaran, 1996). The ability to do so has been attributed to their characteristic to adhere to substrate particles, to produce filamentous cell for penetration and their water activity requirements.Studies on occurrence, co-incidence and levels of amylases in bacteria isolated from soil have been conducted (Dey *et al*., 2002). However, reports on such studies in case of bacteria isolated from mushroom compost are lacking. The bacterial isolates from such compost are found to be well adapted to extremes of environment changes in temperature and pH. Also no work seems to have been reported on using deodar wood dust as substrate for biosynthesis of bacterial amylase enzyme in SSF. Therefore, an attempt has been made to find out the amenability of bacterial strain of *Bacillus* spp. A1 to produce extracellular amylase and to optimize culture parameters in SSF using cheap lignocellulosic solid substrate i.e. deodar wood dust.

RESEARCH METHODOLOGY

The basal salt medium supplemented with 0.5 per cent yeast extract (BSYEM) was used with the following composition (per liter of distilled water) 6.0 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.5 g of NaCl, 1.0 g of NH_4Cl. Separately sterilized solution of 1M $MgSO_4$ (2ml) and 1M $CaCl_2$ (0.1 ml) were added after the medium was autoclaved. The medium was adjusted to pH 10.0.

Culture of *Bacillus* spp. A1, was isolated from mushroom compost by using standard method. The bacterial culture was maintained in liquid medium as well as in solid medium in BSYEM containing 0.5 per cent starch and stored at 4^0C.

Lignocellulosic raw materials used for enzyme production were apple pomace, wheat bran, maize cob, wood dusts of deodar (*Cedrus deodara*) and tune (*Toona ciliata*). These were initially dried under sun followed by drying in oven at 60^0C for 72 h. The dried materials were ground in electric grinder and packed in polythene bags for subsequent studies.

Basal salt yeast extract medium (BSYEM) was used for the production of amylases by *Bacillus* spp. A1. Twenty mililitre of the medium was dispensed into 250 ml Erlenmeyer flasks containing 10 g of substrate and autoclaved at 15 psi for 20 min. The medium was supplemented with 0.5 per cent yeast extract and inoculated with 2ml of bacterial suspension (O.D. 1.0 at 540 nm) and incubated at 35^0C under unshaken conditions. The flasks were gently tapped intermittently to mix the contents. At the desired intervals, the flasks were taken out and the contents were centrifuged at 5300 rpm for 30 min at 4^0C. The culture supernatant was used as crude amylase preparation. Prior to centrifugation, the samples were withdrawn for determining viable number of cells by standard viable count techniques.

The procedure used to estimate amylase activity essentially consisted of estimating reducing sugars formed by the action of amylase on soluble starch. The reducing sugars formed were estimated by calorimetric method of Miller (1959) and the results were extrapolated by the standard curve drawn using maltose.

The reaction mixture contained 0.5 ml of 1 per cent starch solution in bicarbonate buffer (0.1M, pH 10) and 0.1 ml of diluted enzyme and made to final volume of 1 ml with 0.4 ml of bicarbonate buffer and incubated at 55^0C for 15 min.

One unit (u) of amylase activity was defined as the amount of enzyme that produced reducing sugars equivalent to 1 micro mole of maltose per litre per minute under given assay condition. Amylase production is expressed as ug^{-1} dry bacterial deodar dust (DBD). The enzyme assays were performed in triplicates with analytical grade reagents. Data were statistically analyzed by analysis of variance technique (one way classification) following Gomez and Gomez (1976).

RESEARCH FINDINGS AND ANALYSIS

In the SSF process, the solid substrate not only supplies the nutrients to the culture but also serve as an anchorage for microbial cells. Therefore, physical nature and chemical composition of substrate are of critical importance. Several workers have used various lignocellulosic raw materials such as wheat bran, soybean meal, maize bran, rice bran, banana waste, polyurethane foam, wheat flour, corn flour, sweet potato etc. (Bhumibhamon, 1986; Krishna and Chandrasekaran, 1996; Soni *et al.,* 1996) for production of bacterial amylase. Among these lignocellulosic raw materials, wheat bran has emerged as most suitable substrate for amylase production by bacteria in SSF. The universal suitability of wheat bran may be due to the fact that it contains sufficient nutrients in right proportion and its ability to remain loose even in moist state, thus providing larger surface area (Babu and Satyanarayana, 1995).

In the present study, a number of solid substrates (*i.e.* apple pomace, wheat bran, maize cob and wood dust of deodar and tunni) were used for production of bacterial amylase (Table 1). Among the various substrates tested, deodar wood dust proved to be the best and suitable

substrate for amylase production in SSF after 48h of incubation. Minimum amylase activity (94.49Ug^{-1} DBD) was obtained when apple pomace was used as substrate. The production of the enzyme activity was found to be growth associated as maximum amylase activity corresponded to maximum viable count irrespective of substrate used. However, the enzyme yields with different substrates indicate that the amylase production by the strain of *Bacillus* spp. A1 is not primarily determined by the starch content. The higher amylase activity on deodar wood dust may also be the result of synergistic action of various types of amylases elicited by the substrate. The substrates such as apple pomace, maize cob, tuni were found unsuitable for amylase production.

The production of amylase activity on deodar dust can be attributed to the absence of phenomena of catabolite repression as the reducing sugars are not present in the culture supernatant of *Bacillus* spp. A1 unlike the presence of reducing sugars in culture filterate when wheat bran, apple pomace, maize cob and tuni are used as substrates. Substrates with poor enzyme yield exhibiting free sugars have been reported for the production of glucoamylase by *Aspergillus niger* (Pandey and Radhakrishanan, 1993). Amylase production by *Bacillus* spp. A1 was comparatively more on wheat bran as compared to other substrates used (Table 1). This is in agreement with the earlier studies on bacterial amylase production in SSF (Babu and Satyanarayana 1995; Soni *et al.,* 1996). However, Deodar wood dust proved to be a better substrate for amylase production in SSF producing 264.4 ug^{-1} of amylase activity as compared to 233.00 ug^{-1} activity on wheat bran. The difference in the enzyme yield with different substrates indicates that the amylase production by this strain of *Bacillus* spp. A1 is not primarily determined by the starch content. The higher amylase activity on deodar wood dust may also be the result of synergistic action of various types of amylases elicited by the substrate. Deodar dust which supported the production of maximum amylase activity had minimum protein content of 290µg/ml and produced no reducing sugar. The presence of minimum protein indicate the high specific activity of amylase when deodar dust was used as substrate.Results obtained for the optimization of the process parameters for SSF production of α-amylase with deodar dust as substrate demonstrated clearly the impact of the process parameters on the gross yield of enzyme as well as their independent nature in influencing the organisms ability to synthesize the enzyme. Based on the results deodar dust was used as the substrate for production of alkaline amylase by *Bacillus* spp. A1 in all subsequent experiment.

Growth and production of amylase activity was monitored for 72h in BSYEM (Table 2). It was observed that amylase activity was essentially present in the culture

Table 1: Alkaline amylase production by *Bacillus* spp. A1 in solid state fermentation on different types of substrates at 48 incubation

Substrates	Amylase activity* (ug^{-1} DBD)	Reducing sugar (µg/ml)	Viable count (cfu x 10^6)	Protein (µg/ml)	Final pH
Wheat bran	233.0	140.0	124.0	400.0	8.88
Apple pomace	94.49	350.0	102.0	800.0	8.97
Deodar	264.4	0.00	140.0	290.0	9.63
Maize cob	168.5	110.0	82.00	370.0	9.36
Tuni	126.4	80.00	136.0	880.0	9.30
S.E.	0.261	0.516	0.577	0.577	0.004
C.D. (P=0.05)	0.545	1.077	1.204	1.204	0.008

*Reaction mixture: substrate (1% starch in 0.1 M, pH 10, bicarbonate buffer) = 0.5 ml; Enzyme filtrate diluted (0.1 ml); total volume made to 1 ml with bicarbonate buffer; incubation temperature 55^{O}C; incubation period 15 minutes.

Table 2: Time course of alkaline amylase production by *Bacillus* spp. A1 in SSF

Incubation (h)	Amylase activity* (Ug^{-1} DBD)	Viable count (cfu x $10^{6)}$	Protein µg/ml	Final pH
0	210.4	24.00	90.00	10.00
24	222.0	96.00	170.0	9.80
48	264.0	124.00	290.00	9.60
72	262.2	142.00	288.00	9.20
S.E.	0.580	2.582	1.291	0.007
C.D. (P=0.05)	1.338	5.954	2.977	0.016

* Reaction mixture same as in Table 1

supernatant at 0h (210.4 Ug^{-1} DBD) of growth. From this point the level of amylase activity increased gradually over the next 48h.Decrease in the level of enzyme activity was observed at 72h of incubation. It might be due to denaturation and/or decomposition of α-amylase as a result of interactions with other compounds in the fermentation medium (Ramseh and Lonsane, 1987) or due to action by proteases secreted into the system (Zhu *et al.,* 1994).

The maximum cell growth and highest amylase yield of *Bacillus* spp. A1 coincides at temperature of 35^0C, which is identical to that reported for *Bacillus licheniformis* grown on wheat bran (Ramesh and Lonsane, 1989). The results indicate the independent nature of temperature effect irrespective of the type of solid substrate used. Highest amylase titres in bacterial system have already been reported occur at temperature that are optimum for the growth of culture (Krishna and Chandrasekaran, 1996).

The effect of varying the initial pH (7-11) for the fermentation medium (BSYEM) on the production of amylase activity on deodar dust in SSF was studied. It is evidient from Table 3 that amylase production was affected by initial pH of the medium and was found to be growth associated. The amylase activity was not detected at pH below 7.0. On analysis, amylase activity at pH 10.0 (264.2 Ug^{-1}DBD) and 10.5 (264.0Ug^{-1}DBD) was found to be statistically at par with each other while the activity was significantly different at all other pH. The maximum growth (124 cfu x 10^6) and enzyme activity (264.2 Ug^{-1} DBD) was observed at pH 10.0. The fermentation medium with initial pH 10.0 was used for subsequent studies on amylase production in SSF. The initial pH of the medium influenced the yield of amylase production on deodar dust as was reported for wheat bran (Ramesh and Lonsane, 1987) and banana fruit stock (Krishna and Chandrasekaran, 1996). The present results add to the observation that variation in pH influence the efficiency of the organism whatever the type of solid substrate used.

A study of amylase production was conducted at 25,30,35,40 and 50^0C using BSYEM with pH 10.0.The data presented in Table 4 showed that amylase production was affected by incubation temperature. The production of amylase activity initially increased with the increase in temperature from 25 to 35^0C and thereafter, it decreased. The production of amylase activity was maximum at 35^0C (264.4Ug^{-1} DBD) which was also optimum for the growth of the isolate at the end of 48h of incubation. Maximum protein content was found at 35^0C (290µg/ml) which was followed by 170 µg/ml at 40^0C.The maximum cell growth and highest amylase yield of *Bacillus* spp. A1 coincides at temperature of 35^0C, which is identical to that reported for *Bacillus licheniformis* grown on wheat bran (Ramesh and Lonsane, 1989). These results indicate the independent nature of temperature effect irrespective of the type of solid substrate used. Highest amylase titres in bacterial system have already been reported to occur at temperature that are optimum for the growth of culture (Krishna and Chandrasekaran, 1996).

Table 3: Effect of pH on alkaline production by *Bacillus* spp. A1 in SSF at 0 h and 48 h (figures in parentheses) of incubation

Initial pH	Amylase activity* (Ug^{-1} DBD)	Vaible count (cfu x 10^6)	Protein (µg/ml)	Final pH
7	24.00 (48.00)	12.00 (20.00)	51.00 (200.00)	7.00 (7.45)
8	42.20 (67.40)	18.00 (72.00)	74.00 (250.00)	8.00 (8.06)
9	168.20 (222.20)	28.00 (100.00)	82.00 (289.00)	9.00 (8.93)
10	210.40 (264.20)	42.00 (124.00)	90.00 (290.00)	10.00 (9.63)
10.5	208.00 (264.00)	30.00 (104.00)	100.00 (298.00)	10.50 (9.89)
11	102.00 (166.40)	16.00 (80.00)	85.00 (280.00)	11.00 (10.00)
S.E.	0.374	0.577	0.577	0.331
C.D. (P=0.05)	0.772	1.191	1.191	0.676

* Reaction mixture same as in Table 1

Table 4: Effect of temperature of alkaline amylase production by *Bacillus* spp. A1 in SSF at 48 h of incubation

Temperature (^{O}C)	Amylase activity* (Ug^{-1} DBD)	Viable count (cfu x 10^6)	Protein (µg/ml)	Final pH
25	120.20	64.00	130.00	8.65
30	222.20	92.00	130.20	8.89
35	264.40	124.00	290.00	9.63
40	144.20	84.00	170.00	8.40
50	100.00	70.00	130.00	8.20
S.E.	0.372	0.816	0.731	0.008
C.D. (P=0.05)	0.829	1.819	1.629	0.018

*Reaction mixture same as in Table 1

Partial biochemical characterization of alkaline amylase:

The primary goals of an optimally performing detergent α-amylase are high activity and stability in the temperature ranges from 42-60^{0}C under alkaline pH conditions (Kunamneni *et al*., 2005; Hagihara *et al.*,2001; Ara *et al*., 1993). In the present investigation, optimum temperature of enzyme activity was determined by varying the reaction temperature from 40 to 80^{0}C. The result (Fig.1a) showed that amylase was most active at 55^{0}C.There was gradual decrease in enzyme activity when the reaction mixture was exposed to higher temperature which is in accordance with previous report where alkaline amylase having maximum catalytic activity from 55-60^{0}C has been reported for *Bacillus* spp. KSMK-38 and *Micrococcus* sp. (Hagihara *et al*., 2001;Kimura and Horikoshi, 1989).

The temperature relationship of amylases is better described in terms of their thermostability properties. The thermal stability of enzyme was investigated by incubating the enzyme at temperature ranging from 30-90^{0}C for 15 min. The results (Fig. 1d) exhibited that enzyme was stable up to 55^{0}C. Thermal denaturation of amylase started after exposure to temperature above 55^{0}C for 15 min. Activity decreased sharply at 60^{0}C and more than 50 per cent enzyme inactivation occurred at 70^{0}C. Several workers have examined the thermostability of alkalophilic *Bacillus* sp. The amylase is stable at temperature 50^{0}C for 10 min in most of the *Bacillus* sp. like *B. licheniformis*, *B. circulans* CRS13, *B. subtilis* (Padmanabhan *et al*., 1992; Dey *et al*., 2002; Ohdan *et al*., 2000; Ragunathan and

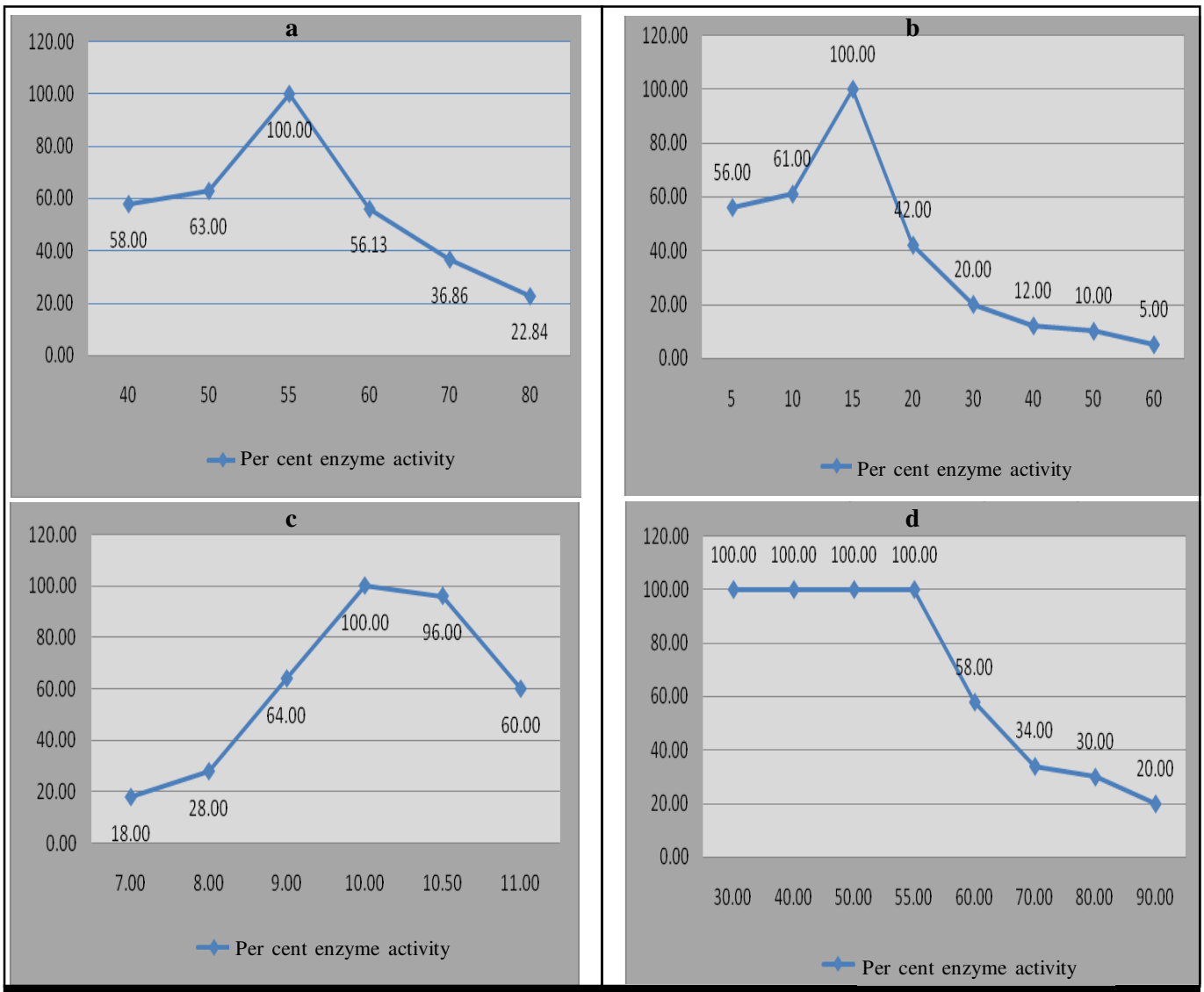

Fig. 1: Effect of (a)temperature (b) incubation period (c) pH on per cent enzyme activity and (d) effect of temperature on enzyme stability

Swaminathan , 2005).

The amylase from *Bacillus* sp. A1 showed activity at the highest pH of 10.0 to 10.5 (Fig. 1C) reported thus far for amylases from microorganisms and higher plants. As mentioned earlier, most of the bacterial amylases are acid or neutral enzyme having pH optima of 6.5 and are essentially useless in detergents because working pH range of detergent is between 8 and 11 (Hagihara *et al*., 2001).The pH optima ranging between 8-9.7 have been reported for some of the alkalophilic bacteria (Kimura and Horikoshi 1989; Lin *et al*., 1998). Hagihara *et al*. (2001) have isolated *Bacillus* sp. strain KSMK-38 from soil sample having maximum amylase activity at pH of 8.0- 9.5. However, all these studies have been conducted by using the process of SmF unlike the present studies in SSF. A high pH optima is especially important in laundry and automatic dishwashing detergents.

Under the optimum conditions of enzyme production and measurement of activity, this strain of *Bacillus* sp. A1 gave 288.2 Ug-1 DBD of alkaline amylase in solid state fermentation of deodar wood dust.

In conclusion, the study indicates the success of solid state fermentation technique for the production of alkaline amylase by alkalophilic *Bacillus* spp. This is the first example where deodar wood dust used as substrate exhibited maximum amylase production. Thus, with the use of chief substrate in the form of deodar dust and higher concentration of product formed, the use of SSF technique for bacterial amylase production will lead to considerable reduction in capital investment.

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