



JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal



Published by
Faculty of
Biotechnology and
Food Sciences

Andualem and Gessesse 2013 : 3 (1) 30-34

ISOLATION AND IDENTIFICATION OF AMYLASE PRODUCING YEASTS IN 'TELLA' (ETHIOPIAN LOCAL BEER) AND THEIR AMYLASE CONTRIBUTION FOR 'TELLA' PRODUCTION

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ARTICLE INFO

Received 28. 12. 2012
Revised 25. 4. 2013
Accepted 29. 4. 2013
Published 1. 8. 2013

Regular article



ABSTRACT

'Tella' is local beer which is used in most part of Ethiopia. It is made from cereals, such as barley, wheat, maize and other crops. *Rhamnus prinoides* is also used to provide a special aroma and flavor as well as antiseptic agent. The objective of this study is to determine the contribution of amylases from tella yeast isolates and compare with the role of amylase from malt. House hold 'tella' samples were collected and plated on starch agar and then amylase positive isolates of yeast were identified by folding iodine solution over the starch agar. Amylase assay and activities were investigated by standard methods and compared with amylase from malt. According to this study, the activity of amylases which was extracted from yeast isolates was very low and may have no contribution in the conversion of starch into fermentable sugars. Thus, it is better to avoid such organisms from 'tella' fermentation in order to discriminate unwanted bio-products. In conclusion, the substrates and ingredients should be sterilized and introduced into the fermentation system aseptically.

Keywords: Amylase, malt, starch, tella, yeast isolates, enzyme activity

INTRODUCTION

'Tella' is a local beer which is widely prepared and used in both rural and urban area in Ethiopia. It is mostly made from cereals, such as barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* Z.) and maize (*Zea mays* L.). In addition to cereals, *Rhamnus prinoides* L. which is known as gesho in Amharic is used to provide a special aroma, flavor and it is also used as an antiseptic agent against beer spoilage bacteria (Shale and Gashe, 1991). Production of alcohol is carried out by *Saccharomyces cerevisiae* in which it is unable to metabolize starch (Nahvi and Emtiazi, 2003; Martin et al., 2001). This is because *Saccharomyces cerevisiae* does not have amylases, which responsible to convert starch into fermentable sugars. Thus, only fermentable sugars particularly maltose is the principal substrate for yeast fermentation. Amylase from different sources has extensive commercial applications in starch liquefaction, sizing in textile industries and paper and detergent manufacturing process (Hewitt and Solomons, 1996; Bajpai and Bajpai, 1998; Lin, et al., 1998; Teodoro. and Martins, 2000). Though amylases originate from different sources, the microbial amylases are the most produced and used in industry, due to their cheap and high productivity (Burhan et al., 2003). Among microorganisms, a number of new amolytic yeast species were isolated from different sources, such as *Candida edax*, *Candida antarctica* and *Sachwanniomyces allauvius* (De Mot and Verachtert, 1987; Ettalibi and Baratti, 1988; Boze et al., 2004).

In brewing process, the principal enzymes responsible for starch conversion are amylases. They can degrade starch into maltose, glucose and other form of carbohydrates. They can convert the available starch to fermentable sugars. The source of these enzymes in many types of beer production is clearly known i.e., originated from malt, while in the process of production of 'tella' the enzyme source is not yet investigated and well documented. In 'tella' brewing process different substrates such as malt, enkuro (made from dry roasted corn which is grounded into fine flour and mixed with water and subsequently cooked using pan) or kita (a bread prepared from flour of grains like corn, wheat, barley separately or in mixture) and derokote (a socked grain such as corn or wheat or barley that roasted on the pan until the color changed into brown) are used as a source of carbon in the fermentation. Malt amylase may not be enough to decompose starch molecules into fermentable sugars from different substrates, since kira, enkuro and derokote are produced from cereals and rich in content of starch. As opposed to commercial beer, 'tella' is not carried out aseptically and different environmental microorganisms introduced into the fermentation system.

Thus different microorganisms from the environmental sources may contribute amylases to increase the efficiency of conversion of starch from malt and adjuncts into maltose and other fermentable sugars.

In this project, fermentation is defined as a process in which a product ('tella') is produced by the mass culture of microorganisms available in the environment. Thus, from mass culture there may be some species of yeasts which can contribute amylases to metabolize starch of malt and adjuncts into fermentable sugars.

The objective of our study was to identify the most efficient isolate of yeasts with a potential to produce amylases for 'tella' production and as well as to analyze the characteristics of their enzyme activities and lastly to compare with the amylase activity of malt. The data generated in this investigation may serve as baseline to design the better 'tella' production method in aseptic condition (Seidman and Moore, 2000).

MATERIAL AND METHODS

Isolation and identification of yeast isolates

'Tella' samples were collected from house-holds in Gondar, using aseptic cupped test tubes. Yeast isolates were isolated according to Min et al., (1999) and Fossi et al (2005). The samples were first enriched on starch broth (1% soluble starch, 0.5% peptone (w/v), 0.55 yeast extract %) at pH 5.5. One ml of 'tella' was mixed with the prepared medium and incubated at room temperature for 3 days with constant shaking at 250 rpm. Two percent of the enriched liquid medium was then spread on starch agar (1% soluble starch, 0.5% peptone, 1.5% yeast extract, 2% agar, 1 gm/l chloramphenicol) plates (Marchal, 1976; Min et al., 1999; Fossi et al., 2005). The plates were incubated at room temperature for 48-72 h until yeast typical colonies obtained. The colonies were further sub-cultured on starch agar plates to get pure colonies. Iodine solution was folded over the surface of the plate in order to select amylase producing isolates. Those colonies having clear zone were selected for further investigation. Morphological and biochemical characteristics of isolates were identified according to the yeast taxonomy (Kreger, 1984; Rippon, 1988; Bajpai and Bajpai, 1998).

Together with the screening of amylase producing yeast isolates, the population density of non-amylase producing and amylase producing yeast isolates were measured by culturing 'tella' samples on malt and starch agar separately. The samples were collected and diluted to 10⁻¹. From each diluted sample, 15 µl

was taken and cultured in triples on starch and malt agar plates and incubated for 3 days at room temperature. Eventually the population density was measured by calculating the colony unit forming of organisms on starch and malt agar.

Enzyme productivity of selected yeast isolates

Selected yeast isolates were cultured at 30°C for 72 hr, in 50 ml of starch broth (1% starch, 0.5% peptone and 1.5% yeast extract) in 250 ml flask and constantly mixed using reciprocal shaker. The pH of the medium was adjusted at 5.5. Two batches were prepared for each isolate: one was used to determine amyolytic activity of the isolated yeast after 90 h fermentation at room temperature and another one used to determine the time course of cell growth and amylase production of each isolate.

From the first batch of fermentation, the yeast cell growth was determined in every 10 hrs interval (Herbert, 1961). The biomass was measured at 610 nm using 1-cm optical cell (Cortez et al., 2005). The remaining supernatant was collected as crude enzyme extract and used to determine the enzyme activity and productivity of the isolate (Cortez et al., 2005).

The broth from the second batch was centrifuged at 6000 rpm for 20 min and the supernatant was collected as crude enzyme extract. A portion of the crude extract was used to determine the enzyme productivity of the isolate by measuring the enzyme activity. The remaining extract was used for characterization of the enzyme activity and stability in different conditions (Keleke et al., 1998).

Malt amylase was extracted from 25 gm malt flour mixed and soaked in 100 ml distilled water. The ratio of malt and water was taken from the local procedure of 'tella' preparation. Malt was first pulverized with grinder and soaked in water for 30 min. The supernatant was separated by centrifuge at 6000 rpm for 15 min and further purified by vacuum filtration. The pure extract which is supposed to be rich in amylase content was used for further investigation together with the amylases extracted from different yeast isolates.

Enzyme assay and characterization

Amylase activity was assayed using starch as substrate (Herbert, 1961; Bajpai and Bajpai, 1998; Keleke et al., 1998; Teodoro and Martins, 2000). The assay was carried out based on the reduction in blue colour intensity due to enzyme hydrolysis of starch (Bajpai and Bajpai, 1998). In this assay, 1 ml enzyme (cell free supernatant) and 10 ml of 1% starch solution were mixed and incubated at 30°C for 10 min. The reaction in the test tube was stopped by adding 10 ml of 0.1N HCl. From this, 1 ml was added to 10 ml iodine solution (0.05% iodine in 0.5% KI). Optical density (OD value) of the solution was measured by spectrophotometer at 660 nm. The same procedure was done using 1 ml-distilled water instead of 1 ml enzyme sample (Bajpai and Bajpai, 1998).

Effect of temperature on amylase activity: The effect of temperature was determined at 20-80°C. One ml of culture extract enzyme was mixed with 1 ml of 1% soluble starch in phosphate buffer 0.005M at pH 6.9 and incubated in a water bath at different temperature for 30 min. The reaction was stopped by using 10 ml of 0.1 N HCl. The OD-value was measured at 660 nm.

Effect of pH on amylase activity: The effect of pH on amylase activity was determined on starch solutions (1%) at pH 3.0-9.0 and 30°C for 30 min. The amylase activity was similarly determined from reduction in blue colour intensity.

Effect of substrate concentrations on amylase activity: Amylase activity of various crude amylase preparations was assayed at various substrate concentrations of 1-9% starch solutions containing 0.005 M NaCl in 0.2 M phosphate buffer at pH 6. (Oboh, 2005).

The comparison between malt and yeast isolates amylase activity: Extracted amylases from each yeast isolate were compared with that of malt amylase. Equal

volume of amylase extract from each yeast isolate was taken and mixed together to compare the cumulative amylase activity with that of amylase from malt at 6.9 pH and room temperature. At 10 min intervals, amylase activity assay was carried out in both cases.

RESULTS AND DISCUSSION

The amylase activity and characteristics of all isolates were determined and compared by using the crude enzyme extract after 72 hrs of fermentation (Fig. 1). Since the distinction among the isolates were laid on the type of biochemical and morphological characteristics of the isolates, it was not possible to define whether amyolytic variation among isolates was the consequence of species variability or environment on the same microorganisms. Thus, the isolates showing high concentration of fermentable sugars i.e., above 70% after 72 hr fermentation were selected for further analysis from 9 isolates (designated as Y1-Y9) (Fig 1). The selected isolates were designated as Y1, Y2, Y3 and Y4. Among selected yeast isolates, Y4 has shown the highest amylase activity (97.6%) in comparison with other selected isolates.

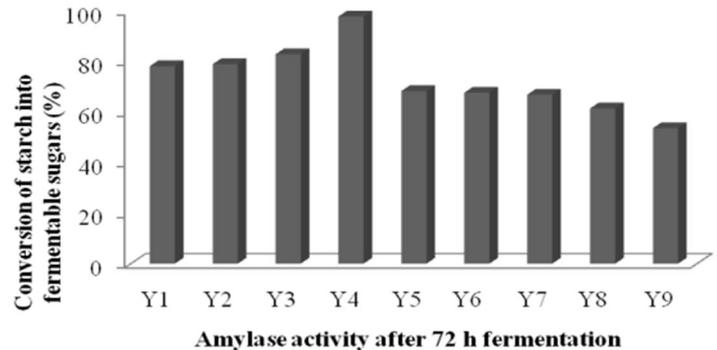
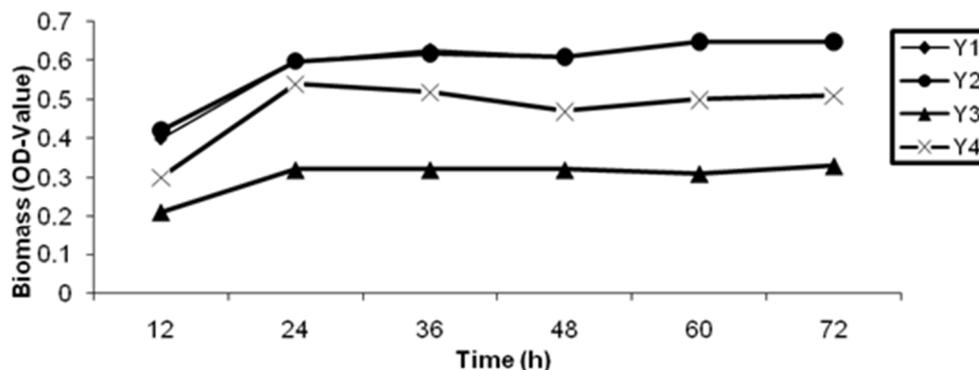
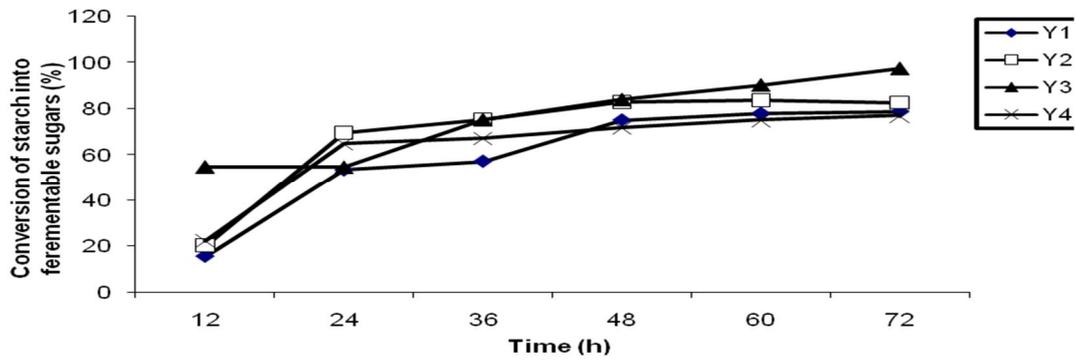


Figure 1 Amylase activity of yeast isolates (Y1-Y9)

Among different amylase positives yeast isolates from 'tella', four isolates (designated as Y1, Y2, Y3 and Y4) were selected for their potential amylase production capacity. As it has been confirmed from morphological, fermentation reaction and assimilation of carbon tests, they were belongs to different species. The colonies producing amylase were surrounded by a distinctive halo or clear zone, indicating that the starch in the medium near the isolates had been hydrolyzed. The production of amylase and growth rate were investigated in the medium with 1% starch as the only source of carbon and 0.5% peptone as a source of nitrogen, which was supplemented with 1.5% yeast extract. The measurement of enzyme activity and cell growth rate of yeast isolates at every 12 h time intervals are shown in Fig. 2. In all isolates, amylase production was increased together with cell mass increment after 24 h of fermentation. The maximum amylase activity was recorded after 24 hrs of fermentation at room temperature and pH 5.5. Among studied isolates, there is no significant variation in growth and enzyme productivity except that isolate Y3 enzyme activity highly increased after 36 h of fermentation. With regard to biomass, isolate Y1 and Y2 were comparatively greater than other isolates while the cell growth of Y3 was lower than other isolates. To produce high amount of amylase with high enzyme activity, over 24 h of fermentation is important.





B) **Figure 2** Time course of cell growth and amylase activity of 4 yeast isolates (A = cell growth and B = amylase activity)

The biomass density of non-amylase producing and amylase producing yeast isolates were determined by culturing ‘tella’ samples on malt and starch agar separately. The colony forming unit (cfu) of amylase producing yeast isolates on starch agar was 2.2×10^3 while that of yeast isolates cultured on malt agar was 2.6×10^4 . According to this finding, the population density of non-amylase producing yeast isolates was greater than that of amylase producing yeast isolates. This may be due to the presence of alcohol producing yeast (*Saccharomyces cerevisiae*) isolates in malt agar (data was not shown). Amylase extracted from selected yeast isolates and malt was incubated with 1% soluble starch solution for 60 min at 30 °C (Fig. 3). Amylase activity was

measured at every 10 min intervals. Amylase from Y3 was shown optimum activity i.e., over 94% after 20 min of incubation. In the rest isolates, rate of conversion of starch into fermentable sugar was gradually increased after 10 min of incubation. On the other hand, the cumulative activity of amylases that extracted from all yeast isolates is very low in comparison with that of the amylase of malt in origin. The amylase activity of malt was very high (100%) after 10 min of incubation. According to this investigation, amylase from malt may be enough to degrade starch from malt and other adjuncts in ‘tella’ production.

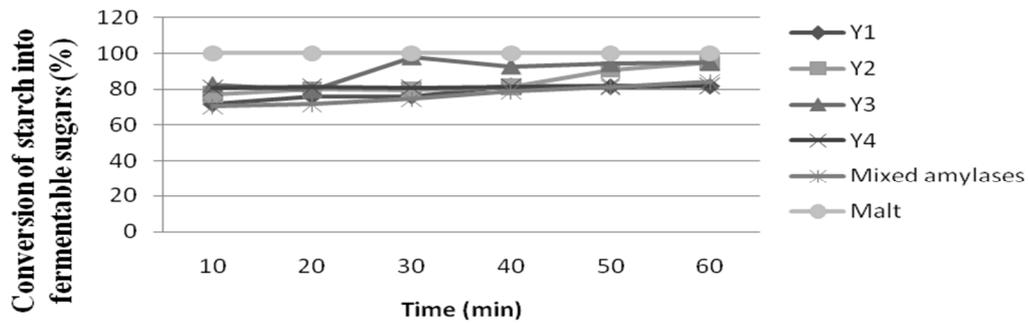


Figure 3 Starch converted into fermentable sugars at 30°C at 10 min interval of incubation

The amylase activity from isolate Y2 and Y3 was gradually increased after 30°C of incubation. The optimum temperature for malt amylase activity was found to be between 10 and 70 °C (Fig. 4). The optimum temperature of amylase activity recorded in the present study was lower than that of amylase isolated from AB3 in starchy soils and *Bacillus lentus* (Alli et al., 1998) but similar to some known

fungi amylase, such as *Apergillus niger*, *Aspergills orizea*, *Mucor pusillus* (Fogarty, 1983). In this study, there was no observed negative temperature effect up on amylases extracted from yeast isolates and malt and could potentially be exploited in industrial activity that requires a wide temperature rang. However, the activity of amylase extracted from yeast isolates was very low in comparison to amylase from malt.

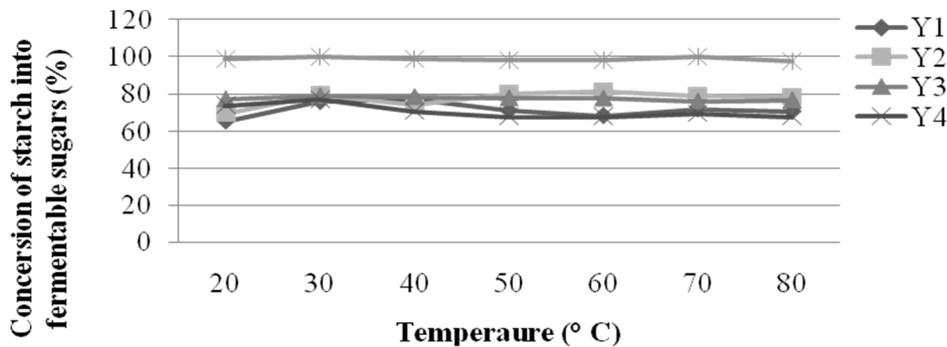


Figure 4 Effect of temperature on the activity of amylases obtained from yeast isolates and malt

The effect of pH on the enzyme activity has shown (Fig 5). High amylase activity (72.2-76.1%) of isolate Y1 was extended from pH 5 to pH 9. The amylase activity of isolate Y2 was reduced from 77.9% to 63% with the range of pH 3-8 and even highly reduced at pH 9 with the enzyme activity of 46.3%. The optimal enzyme (amylase) activity (79.4%) of isolate Y3 was at pH 6, while that of

isolate Y4 amylase activity was 82.1% at pH 8. The amylase activity of malt was extended from pH 3 to 7 with 98.8-99.98% of conversion of starch into fermentable sugars. Except isolate Y2, the amylase activity of other isolates were in line with the optimal pH for certain fungal (*Aspergillus flavus* and *M. pusillus*) amylase activity reported by Alli et.al (1998).

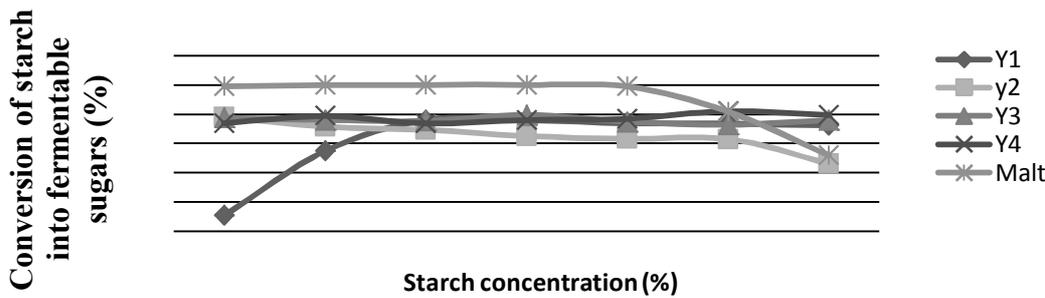


Figure 5 Effect of pH on the activity of amylases obtained from yeas isolates and malt

As this study suggests that all enzymes would be useful in the process that requires wide range of pH change from acidic to neutral range and vice-versa. However, there was drastic decline in the activity of the enzyme produced from malt and isolate Y2 after pH 7 and 8, respectively which is in line with other reports (Hyun and Zeikus, 1985; Eke, and Oguntimehin, 1992). As oppose to the above, some amylases extracted from Y1, Y3 and Y4 showed some residual activity at pH 9 which is in agreement with Alli et. al. (1998) report on the amylase from *A. flavus*, *Aspergillus niger*, *Rhizopus oryzae* and *M. pusillus*.

As shown in Fig 6, in all yeast isolates, there was a high enzyme activity in the range between 1.0 to 3% of starch concentration. But there was a slight decline in the amylase activity from 4 to 9% starch concentration. This report is in line with that of Alli et al. (1998) as well as Oboh (2005) report. Malt amylase activity was only reduced from 99% to 85.6% from the range of 1 to 8% starch substrate, respectively but the enzyme activity was highly reduced at 9% starch concentration. According to this study, to have optimal conversion of starch into fermentable sugars, the concentration of starch may not be exceeds over 3%.

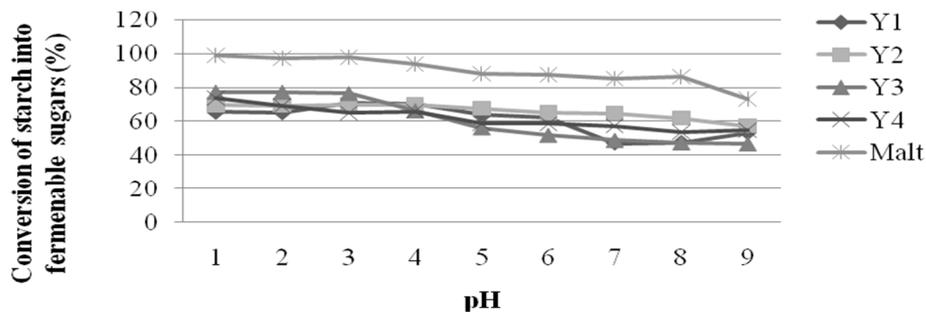


Figure 6 Effect of substrate (starch) concentration on the activity of amylases extracted from

According to this investigation, since the activity of amylases that extracted from yeast isolates is very low in comparison with the amylase obtained from malt, they may not be enough to convert starch from ‘tella’ substrates efficiently into fermentable sugars. These yeast isolates may be considered as wild yeast contaminants and thus it is better to avoid such organisms from ‘tella’ fermentation in order to discriminate scum formation and foul smelling. Thus, the ingredients (gesho, malt and adjuncts) should be sterilized in order to minimize source of undesired wild yeast contaminants in the process of ‘tella’ production.

CONCLUSION

To produce ‘tella’, the substrates should be prepared in a sterile medium and introduced into the system aseptically. Under controlled conditions, the starch should be converted to fermentable sugars by the help of amylase from malt. Therefore, the whole process requires a closely controlled environment since amylases exhibit great sensitivity to the change in temperature, pH and even concentration of substrates. Unnecessary environmental organisms introduced into the fermentation process may be reduced the quality of ‘tella’. Yeasts other than *Saccharomyces cerevisiae* in the process of ‘tella’ production should be avoided since their role to convert starch into fermentable sugar is low in comparison with amylase from malt.

Acknowledgments: We are grateful to Applied Biology and chemistry Departments’ Laboratory technicians, particularly, namely. Ligabaw Worku, Mengistu Endris, Mulugeta Leggesse and Betsegaw Lemma for their professional help. At last not least, I would like to thank to Mulugeta Aemero for his professional advice during investigation.

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