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Functional Foods Enhanced with Microbial Antioxidants

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Abstract: Free radicals plays an important role in the origin of numerous diseases including both lifestyle diseases and physiological diseases like high blood pressure, cancer, diabetes, cardiovascular, neurodegenerative etc. Free radicals act by catalyzing the various toxic oxidative reactions that lead to the formation of toxic lipid peroxides. They also inhibit the enzymes of respiratory chain of mitochondria and damage DNA and proteins which is lethal for the cell. It is therefore important to search for newer alternatives to allopathic medicines with reduced or no side effects. There are numerous medicinal plants that have been reported to possess strong antioxidant activity along with their free radical scavenging activity. Besides plants, various microbes including bacteria and fungi also possess powerful antioxidant activity. Some of the microbes belong to the probiotic group that has the potential to protect the body from the dangerous free radicals. Thus the present paper aims to discuss about the use of various microorganisms as antioxidants and also to develop the unique functional foods fortified with microbial antioxidants for scavenging the free radicals thereby preventing many diseases.

Key words: Antioxidants · Microorganisms · Free Radicals · Functional Foods · Diseases

INTRODUCTION

Free radicals are defined as those reactive chemicals that have an unpaired electron in their outer orbit [1]. Whereas reactive oxygen species (ROS) comprises both free radical and non-free radical oxygen containing molecules such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), singlet oxygen ($1/2O_2$) and the hydroxyl radical (OH). There are various studies proved that these free radicals are responsible for the various diseases like cancer, diabetes, ageing, cardiovascular diseases, autoimmune disorders, neurodegenerative diseases etc. Free radicals also catalyzes a wide range of toxic oxidative reactions like initiation of the per-oxidation of the membrane lipids that leads to the accumulation of lipid peroxides, inhibits mitochondrial respiratory chain enzymes, causes fragmentation or random cross linking of molecules like DNA, enzymes and proteins which ultimately leads to cell death. Free radicals can also destroy the naturally occurring antioxidant enzymes like superoxide dismutase, catalase and peroxidase; causing destruction and lethal cellular effects (e.g. apoptosis) through oxidization of membrane lipids, cellular proteins, DNA and enzymes and shut down cellular processes [2].

There should be balance between the production and scavenging of ROS. Excessive intake of synthetic antioxidants is also injurious to health as some ROS are signaling molecules in some important cellular signaling pathways. Therefore the level of both ROS and antioxidants should be optimal as both extremes are damaging. Thus there is a need for accurate determination of individual's oxidative stress levels before prescribing any synthetic antioxidants supplement [3].

Recent research supports many findings that seem to validate the benefits of food or food components to the promotion of health. It has been widely stated that people who consume a wide variety of food containing some phyto-pharmaceutical like carotenoids, fibers, flavonoids, fatty acids, phytoestrogens, vitamins and minerals, among others, show a reduced risk of developing some diseases and tend to have a better health. As an example, the use of antioxidants provides protection against harmful free

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radicals, usually associated with cardiovascular disease, cancer, diabetes, Alzheimer's disease and age-related functional decline [4].

The various synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT) are available but due to their health hazard, they are used under strict regulation. Thus interest in the use and development of natural antioxidants arises; for instance, α-tocopherol and ascorbic and hydrolyzed proteins from many animal and plant sources [5].

Other antioxidants naturally present in foods are vitamins C and E, carotenoids, flavonoids and other polyphenols. They can serve as potentially useful candidates for functional ingredients. Plant foods which contain these substances such as berries (e.g. cranberry, blueberry, goji, acai), mangosteen, pomegranate, tomato and grapes are now being explored by the functional food industry as potential antioxidants [6].

Besides, the plant sources of the antioxidants like apple juice, blueberry, cider, cranberry, grape fruit, lemon, lettuce, coffee beans, orange, peach, pear, cherry, potato, spinach, tea; there are certain microorganisms particularly bacteria that are reported to possess antioxidant activity. These microbes possess tremendous potential to be utilized as sources of antioxidant compounds which can be incorporated to develop a functional food or a nutraceutical [7].

Mushrooms are also known to be a good source of antioxidant compounds. Apart from their nutritional potentials, mushrooms are also sources of physiologically beneficial bioactive substances that promote good health. Health promoting properties, e.g. antioxidant, antimicrobial, anticancer, cholesterol lowering and immuno-stimulatory effects, have been reported for some species of mushrooms [8- 10]. Both fruiting bodies and the mycelium contain compounds with wide ranging antioxidant and antimicrobial activities [9,11].

Antioxidants from Microbial Source: Antioxidants from microbial source were identified as early as in 1980s; however the relationship between microorganisms and antioxidants was established only in the beginning of this century [12, 13]. Since then, large number of microorganisms has been identified as the source of antioxidants.

In a study, about nine genera of bacteria, including both Gram-positive and Gram-negative were found to produce the compound with antioxidant activity. The maximum activity was found in the methanol fractions of three *Pseudomonas* species [7]. Besides bacteria, some fungi, few Actinomycetes and yeasts were also found to produce the compounds with antioxidant activity. Zaika and Smith [14] found that *Streptomyces* species produce certain bioactive compounds with antioxidant activity when it was added to the lard. It was also found that brown and yellow pigments produced by mycelium of *A. niger* have antioxidant properties. It was concluded that the antioxidant activity of the extracts might be due to their synergistic effect.

In another study, some fungi have been also identified as natural source of antioxidants. The ethyl acetate extracts of several *Penicillium, Aspergillus* species, *Rhizopus oryzae* protected linoleic acid better than the control [15]. The ethyl acetate extracts obtained from broth and mycelium produced similar antioxidant activity. Further studies revealed that the production of antioxidants by *Aspergillus candidus* CCRC 31543 was enhanced by the addition of sucrose or lactose and ammonium sulphate and the extracts exhibited high free radical scavenging activity as compared to the α-tocopherol [16].

About 750 species of filamentous fungi were also isolated and screened for their antioxidant activity. [17]. Two antioxidants namely citrinin and protocatechuic acid were isolated and identified from the filamentous fungi. Another antioxidant c urvulic acid was also isolated from an unidentified *Penicillium.* All the above three compounds exhibited good antioxidant activity. The curvulic acid had the highest AOA followed by the curvulic acid methyl ester, protocatechuic acid and citrinin.

The antioxidant property of hot water extract (WE) obtained from *Dictyophora indusiata* was found to have free radical scavenging ability on DPPH (97.35% at 2 mg/ml concentration). The reducing power of WE was moderate (1.22 at 2 mg/ml). Similarly, the WE displayed average scavenging effect on hydroxyl radical (52.28% at 2 mg/ml) and superoxide anion scavenging effect (48.64% at 2 mg/ml). However, the WE exhibited a very weak ferrous ion chelating effect of 18.56% at 2mg/ml concentration [18].

In another study, isolated fungi from soil of different areas of Punjab, India for AOA by dot blot assay showed around 45% of fungal isolates having antioxidant potential. Two selected strains of *Aspergillus* spp (PR78 and PR66) showed quantitatively best antioxidant activity b y DPPH assay were further tested for their reducing power, ferrous ion and nitric oxide ion scavenging activity, FRAP assay and total phenolic content. Different physio-chemical parameters were optimized for enhancement of the activity. This revealed stationary culture grown for 10 days at 25°C at pH 7 to be the best for AOA. Sucrose in the medium as carbon source resulted in highest AOA. Sodium nitrate, yeast extract and peptone were good sources of nitrogen but sodium nitrate was the best among these. The extraction of the broth culture filtrates with different solvents revealed ethyl acetate extract to have best AOA. The activity as expressed by ethyl acetate extract of *Aspergillus* PR78 was equally effective as that of commonly used antioxidant standard, ascorbic acid [19].

The AOA of organic extracts of eight fungal species, *Ganoderma lucidum, Ganoderma applanatum, Meripilus giganteus, Laetiporus sulphureus, Flammulina velutipes, Coriolus versicolor, Pleurotus ostreatus* and *Panus tigrinus*, was evaluated for free radical (DPPH• and OH•) scavenging capacity. The highest DPPH• scavenging activity was found in the methanol extract of *G. applanatum* (12.5 μg/mL, 82.80%) and the chloroform extract of *G. lucidum* (510.2 μg/mL, 69.12%). The same extract also showed the highest LP inhibition (91.83%, 85.09%) at 500μg/mL, while the methanol extracts of *G. applanatum* and *L. sulphureus* showed the highest scavenging effect on OH• radicals (68.47%, 57.06%, respectively) at 400μg/mL. The anti-oxidative potencies correlated generally with the total phenol content (0.19-9.98 mg/g). The HPLC determination showed that the majority of analyzed species contained gallic and protocatechic acids. Consequently, these fungi are shown to be potential sources of anti-oxidative agents [20].

Attempt has also been made to evaluate the AOA of fungal endophytes inhabiting *Emblica officinalis* keeping in view their medicinal importance of the selected host plant in Indian traditional practices. A total of four endophytic fungi belonging to Phylum Ascomycetes were isolated from different parts of the plant which were characterized morphologically and by using rDNA-internal transcribed spacer. The most frequently isolated endophyte was *Phomopsis* sp. The antioxidant activity by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power assay and total phenol were evaluated using ethanolic extract of endophytic fungi. DPPH activities in all the ethanolic extract increased with the increase in concentrations. Endophytes, *Phomopsis* sp. and *Xylaria* sp. showed highest antioxidant activity and also had the higher levels of phenolics [21].

In general, *Aspergillus* species were found to be the effective producers of antioxidant compounds. This hypothesis was proved by Esaki *et al*. [22] when about 30 strains of *Aspergillus* were evaluated for their antioxidant activity by using methanol extracts of fermented soybeans (MEFS) that prevented oxidation of methyl linoleate. It was found that MEFS of 28 strains possess better antioxidant activity than the non-fermented soybean; besides all the *Aspergillus* strains were better than the control. The MEFS obtained from *A. saitoi* had the highest antioxidant activity; it was also concluded that the MEFS of 4-days incubation period gave higher antioxidant activity than MEFS of shorter days incubations.

The component 2, 3-dihydroxybenzoic acid was identified as the active fraction in MEFS and the highest concentration was found in the 4 day incubated samples. This compound was also identified in cultures of *Penicillium roquefortii* IFO 5956 [23].

The methanolic extracts of fermented soybean foods like miso, natto and tempeh were evaluated for their antioxidant activity. It was concluded that the antioxidant activity of tempeh was most effective followed by miso. The antioxidant activity of methanolic extracts of natto was less than that of other fermented products but was equivalent to that of unfermented soybeans. Another natural antioxidant compound 3-hydroxyanthranilic acid has been identified in tempeh that exhibit strong antioxidant activity in soybean oil and soybean powders. Hoppe *et al*. [24] isolated and identified antioxidant compound 5-(δ-tocopheroxy)-δ-tocopherol from tempeh fermented by *Rhizopus oligosporus*.

These findings suggest that fermentation by mould cultures is more active in producing antioxidants than the bacterial (e.g. *Bacillus natto*) cultures.

Another antioxidant compound called gallic acid (type of phenolic acid) is found in many natural sources including microbial products. Gallic acid has been isolated from cultures of *Penicillium* and *Aspergillus* [25]. The highest amount of gallic acid was produced by *Aspergillus terreus* S-4 amongst 98 strains of soil organisms tested. The antioxidant compound was produced on a complex media that significantly improved the organism's ability to produce gallic acid compared to basal medium and Czapek-Dox medium. Another antioxidant compound 'protocatechuic acid' was also produced by *Aspergillus terreus* S-4.

2,2-Methylenebis (5-methyl- 6-*tert*-butyl-phenol) has been identified as an antioxidant from the fungus *Penicillium janthinellum*.

The fungus belonging to genera *Eurotium* has been found to produce several antioxidants [26]. The main antioxidant compounds identified were dihydroauroglaucin, auroglaucin and flavoglaucin that was produced by *E. chevalieri* IFO 4086 and *E. repens* IFO 4041. Besides, *Aspergillus chevalieri* also produced all three antioxidants while *Penicillium charlesii* produced only flavoglaucin.

Amongst Actinomycetes, *Streptomyces* sp. USF-319 produces three radical scavenging antioxidants out of which one inhibits 5-lipoxygenase [27]. The antioxidant compounds produced include mycotrienin II, trienomycin A and trienomycin B; which are identical to ansamycin antibiotics. Mycotrienin II was the most active compound of the three, but was considered a moderate antioxidant compared to synthetic antioxidant butylated hydroxytoluene (BHT). However, mycotrienin II was found to inhibit 5-lipoxygenase.

Similarly, *Streptomyces* sp. USF-142 produced antioxidant compound 3-(2-methylphenyl) propionamide-3-one based on the reduction of 2, 6-dichlorophenol indophenol to leuco indophenol. Its reducing activity was similar to that of ascorbic acid, 3-(2-methylphenyl) propionamide-3-one was not found to have AOA in linolenic acid. The researchers believed the lack of AOA to the fact that the molecule would exist as the keto form, thus no OH would be available to participate in the AOA [27].

Another antioxidant compound atroventin was isolated from *Penicillium paraherquei* and was found to have good AOA [28]. *Streptomyces chromofucus* and *Streptomyces prunicolor* produced carazostatin and 7-demethylnaphterpin, respectively, as free radical scavengers [29, 30].

Some other antioxidants like 2, 4-dihydroxy-3, 5, 6-trimethylbenzene (DHTMB), 2, 4-dihydroxy-3, 5, 6-trimethylbenzoic acid (DHTMBA), *N*-(4,6-dihydroxy-2,3,5-trimethylbenzoyl) glycine (DHTMBG) and its methyl ester (DHTMBE) were isolated from an unidentified *Mortierella* species. The free radical scavenging activity of DPPH radicals by DHTMB and DHTMBA was greater than that by BHT or α-tocopherol while DHTMBG and DHTMBE were less active while all the compounds had similar AOA in linoleate emulsion system [31].

Another group of antioxidants like carotenoids are also synthesized by microorganisms. Beta-carotene from *Blakeslea trispora* and *Duniella salina* and lycopene from *B. trispora* and *Streptomyces chrestomyceticus* subsp. *rubescens* were approved for human food as colouring agent [32]. Similarly, astaxanthin produced by *Xanthophyllomyces dendrorhous* has been approved for use in fish foods. Astaxanthin and lycopene were found to have excellent singlet oxygen quenching activity [33]. It was found that astaxanthin has 10 times greater antioxidant activity than that of lutein, â-carotene, zeazanthin and canthaxanthan [34]. The activity of several carotenoids like lutein, â-carotene and astaxanthin are confirmed using a new fluorometric assay [35].

The most promising microbial strains for production of carotenoids are *B. trispora* and *X. dendrorhous* (formerly *Phaffia rhodozyma*). It was also found that the addition of Span 20 in *B. trispora* culture broths enhanced the production of â-carotene from 0.15 g/L to 2.16g/L [36, 37]. Alkaline conditions with pH as high as 10 is also responsible for increased production of â-carotene from *B. trispora* [36].

The addition of tobacco dust can enhance lycopene production as it prevents the cyclization of the lycopene to â-carotene. Alternatively, pyridine derivatives were found to enhance lycopene synthesis with 2-amino- 5-methylpyridine having a greater stimulating effect than 2-amino-6-methylpyridine [38]. It was also found that carotenoids production can be further enhanced when *X. dendrorhous* was cultured on corn wet milling co-products compared to culturing on yeast-malt extracts [39]. In addition, hydrolyzed wood is a good substrate for *X. dendrorhous* [40, 41]. The levels of total carotenoids and astaxanthin were increased four times. Thus it can be safely concluded that the production of natural antioxidants through microbial fermentation hold a great promise in the coming future except that more research is needed on their optimization of the fermentation process.

The coloured pigment produced by *Staphylococcus aureus* also produces a series of carotenoids through a different biosynthetic pathway [42]. Carotenoids produced in dietary fruits and vegetables are well known for their antioxidant activity by virtue of their free-radical scavenging properties and ability to quench singlet oxygen [43]. It has been shown that *Staphylococcus aureus* can utilize its golden carotenoids pigment to resist oxidant-based clearance mechanisms of the host innate immune system.

Another class of enzymatic antioxidants includes the superoxide dismutases, the glutathione peroxidases (GSHPx) and catalase [44].

Superoxide dismutases (SODs) catalyzes the conversion of superoxide anions to dioxygen and hydrogen peroxide; the latter being broken to water by catalase or peroxidase. SOD neutralizes superoxide ions by going through successive oxidative and reductive cycles of transition metal ions at its active site [45]. Most organisms, microorganisms, plants and animals have at least one SOD [46]. One of the exceedingly rare exceptions is *Lactobacillus plantarum* and related lactobacilli, which use a different mechanism [47, 48]. It is widely accepted that a plant-based diet with high intake of fruits, vegetables and other nutrient-rich plant foods may reduce the risk of oxidative stress and associated diseases [49]. SODs were also produced efficiently by many microbial species [50, 51]. Aerobic microorganisms represent an excellent source for production of superoxide dismutases.

Amongst many aerobic microorganisms, *Corynebacterium glutamicum*, an industrial relevant producer of amino acids and vitamins, is considered as a potent source of SODs. Its high need of oxygen during amino acid production nominates it to have a hyper antioxidant defense system including production of abundance SOD [52,53]. Cloning techniques has been used successfully with many corynebacterial genes [54- 57]. Thus it would be interesting to enhance SOD production using cloning strategies. In addition other microbial species should also be considered for extraction of different superoxide dismutase types.

The probiotic bacteria *Streptococcus thermophilus* has been proven to possess powerful antioxidant activity (AOA), protecting the body from dangerous free radicals which increase in the body due to ageing, stress, sugar, antibiotics and other chemicals and toxins. It also possesses anti-tumour activity especially against colon cancer cells. The probiotic bacteria also help in recovery from malnutrition due to short-term fasting and reduce the associated intestinal atrophy in animal studies [58].

Studies showed that the antioxidant activity can also be developed during fermentation with dairy starter cultures. However, the development of antioxidant activity is strain-specific characteristic. The development of radical scavengers is more connected to the simultaneous development of proteolysis whereas lipid peroxidation inhibitory activity is related to bacterial growth. However, high radical scavenging activity is not directly connected to the high degree of proteolysis [59].

In a study, group of selected lactic acid bacteria were used for sourdough fermentation of various cereal flours for the production of antioxidant peptides. The results showed that the radical scavenging activity of water/salt-soluble extracts (WSE) from sourdoughs was significantly higher ($P<0.05$) than that of chemically acidified dough. The highest activity was found for whole wheat, spelt, rye and kamut sourdough. Almost the same results were found for the inhibition of the linoleic acid auto-oxidation.WSE extracts were confirmed by Reverse Phase Fast Protein Liquid Chromatography. Thirty-seven fractions of WSE extract were collected and assayed under *in vitro* conditions. Almost all the sequences shared compositional features which are typical of antioxidant peptides. All the purified fractions showed *ex vivo* AOA on mouse fibroblasts when artificially subjected to oxidative stress. This study proves the capacity of sourdough lactic acid bacteria to release peptides with antioxidant activity through proteolysis of native cereal proteins [60].

In an another study, the anti-oxidative effect of both intact cells and cell-free extract of *Lactobacillus casei* subsp. *casei* SY13 and *Lactobacillus delbrueckii* subsp. *bulgaricus* LJJ, isolated from the traditional yogurt, was evaluated by various antioxidant assays. The results showed that two *Lactobacillus* strains had good antioxidant capacity, inhibiting the peroxidation of linoleic acid by 62.95% and 66.16%. The cell-free extract showed excellent scavenging superoxide anion and hydroxyl radicals activity. The intact cells on 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) radical scavenging ability and chelating ferrous ion capacity were superior to cell-free extract. The highest reducing activity was equivalent to 305 and 294μM L-cysteine. This study suggests that two strains are high anti-oxidative bacterial strains. Anti-oxidative property of lactobacilli would be useful in the dairy manufacturing industry. They could beneficially affect the consumer by providing dietary source of antioxidants or by providing probiotic bacteria with the potential of producing antioxidants during their growth in the intestinal tract [61].

In an another study by Nedelcheva *et al.* [62], probiotic strain *Lactobacillus plantarum* that was used as a starter culture in the production of dried fermented meat products was assessed for its AOA against peroxide radicals. It was found that the addition of this culture provided the desired fermentation process in the raw sausage mass and reduced the pathogenic flora.

The application of starter cultures with AOA preserved the colour of the meat products and delivered substances with AOA for the organism as well [62].

Similarly, Miang, a kind of traditional fermented tea leaves, contains several kinds of *Lactobacilli spp.* was investigated for its AOA. The antioxidant study suggested that both *L. fermentum* FTL2311 and *L. fermentum* FTL10BR strains could liberate certain substances that possessed AOA expressed as trolox equivalent antioxidant capacity (TEAC) and equivalent concentration (EC) values for free radical scavenging and reducing mechanisms, respectively [63].

Some bacteria are also used to promote the AOA of the cooked foods. In a recent study, the AOA and phenolic contents of Thai *thua nao* was determined. Methanolic extracts of cooked non-fermented soybeans (CNF) and *thua nao*-fermented soybeans prepared by naturally occurring bacteria (TNMX) and *Bacillus subtilis* TN51 (TNB51) were prepared and used to determine antioxidant activity. The results indicated that production process and bacterial fermentation are responsible for improvement of total phenolics and antioxidant activity [64].

Designer Foods as Vehicle for Antioxidants: An important direction in the development of functional foods is the combination of numerous ingredients to achieve a specific set of goals, rather than efforts to uncover the potential benefits of a single food source. Infant formula was probably the first area for designer foods of this type, because of the profound influence of nutrients on the developing brain and immune system. The addition of Docosahexaenoic acid (DHA) to infant formula for enhancing brain and visual development, the alteration of allergenic components in food and the possible use of probiotics and nucleotides to enhance immune response are important developments in this area [65].

Designer foods can also be used as an effective vehicle for the delivery of antioxidant compounds. As discussed in the above paragraphs, the fermentation process with selective probiotic bacteria enhances the level of antioxidant compounds, these types of so called designer foods can be used as an effective mode for consuming the antioxidant rich compounds.

Sports nutrition is another established arena for designer foods. Specific nutritional measures and dietary interventions have been devised to support athletic performance and recuperation. Oral rehydration products for athletes were one of the first categories of functional foods for which scientific evidence of benefit was obtained. Oral rehydration solutions must permit rapid gastric emptying and enteral absorption, improved fluid retention and thermal regulation, to enhance physical performance and delay fatigue. Carbohydrates with relatively high glycemic index combined with whey protein concentrates or other sources of branched chain amino acids have been shown to enhance recovery of athletes. Caffeine, creatine, ribose, citrulline, L-carnitine and branched chain amino acids have each been shown to improve exercise performance or diminish post-exercise fatigue. Whether combinations of these ingredients, blended into foods or beverages, will perform better than the individual ingredients will help to determine the design of future sports foods [6].

Optimal cardiovascular health involves prevention of excessive levels of oxidant stress, circulating homocysteine, cholesterol, triglycerides and fibrinogen and protection of the vascular endothelium. A mix of ingredients that may supply all of these effects could consist of soy protein powder, oat beta-glucan, plant sterols and stanols, folic acid, L-arginine, DHA, magnesium and red wine or green tea polyphenols. Evidence suggests that addressing multiple nutritional influences on cardiovascular health will be more beneficial than addressing only one influence, but more definitive studies are needed [66]. Genetic factors may need to be incorporated for designer foods to achieve their full potential. Polyunsaturated fatty acids, for example, raise the serum concentration of HDL-cholesterol among individuals who carry the Apo A1-75A gene polymorphism, but reduce HDL-cholesterol levels of individuals who carry the more common Apo A1-75G polymorphism [67].

CONCLUSION

With the ever-changing lifestyle of humans, the antioxidant defense systems are often overloaded resulting in oxidative stress. Moreover, the levels of antioxidant defense mechanism decrease appreciably with age. These may result in the development of many diseases. Hence research over the past several decades have primarily focussed on identifying various sources of antioxidants and their consumption in the form of nutraceuticals and functional foods. Antioxidant products may either function intrinsically to scavenge free radicals (e.g. vitamins, PUFA) or specifically stimulate the body's defense system. This review reflects the potential of microorganisms including the probiotics in playing a key role as a good source of natural antioxidant compounds.

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