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# ENRICHMENT OF NUTRITIONAL VALUE OF CORNMEAL WITH PROTEIN AND PUFA USING OLEAGINOUS MARINE YEASTS IN SOLID STATE ERMENTATION.

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## ABSTRACT

The marine yeasts, *Candida tropicalis, Candida parasilopsis, Candida orthopsilosis,* and *Rhodotorula muciliginosa* were isolated from sea waters of Konkan regions and were tested for their ability to grow on cornmeal and enrich it with Single cell protein (SCP) and Polyunsaturated fatty acids (PUFA). The cornmeal was supplemented with either dextrose (10g%),malt powder(1g%), flaxoil (1g%), glycerol (4g%), whey (50%v/v) or sodium acetate (10g%), subjected to Solid State Fermentation (SSF). All the bioproducts, showed increase in total lipids and PUFA, especially with flaxseed meal supplement yielded PUFA≥50%. The marine yeasts under study successfully converted glycerol supplemented cornmeal, to a bioproduct containing gamalinolenic acid (GLA).The bioproduct formed using whey (50%) supplemented cornmeal by marine yeasts in SSF accumulated 11-18% protein and PUFA in the range of 44-48%. *Candida tropicalis* yielded highest protein, 18.9 % on media containing glycerol. The fermented corn meal enriched by SCP and PUFA could be used as animal feed supplement.

**KEYWORDS**: **marine, oleaginous yeasts, SCP and PUFA, SSF, feed supplement,**

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## INTRODUCTION

Production of microbial feed fortification products by solid-state fermentation have significant prospects. The bioproduct produced by microbial fermentation is nutritionally enriched and can be used as feed with improved nutritional value. Several food materials as well as the spent grains have been enriched for better protein, fats and micronutrients[1,2,3]. Increase in the food value of the various grain powders can be obtained by increasing their protein content through fermentation processes which use fungi[4] and thus enrich the feeds. *Trichoderma viridae, Saccharomyces cerevisiae, Geotrichum candidum, Rhizopus oryzae, Penicillium fonculosum, Fusarium solani and Aspergillus niger*, etc., have been reported to enrich various substrates with protein[5,6].Solid-state fermentation of soybean residue, cassava residue, maize meal, fish meal, molasses, etc. to produce pelleted feedstuffs with enriched nutrients is reported[5,6]. It has been shown that, under appropriate conditions, filamentous fungi are able to transform variety of cheaper substrates into high value products containing long chain polyunsaturated fatty acids (LCPUFAs). Harsulkar et al[7] demonstrated bioconversion of flax alpha linolenic acid (ALA) into Eicsapentaenoicacid (EPA) and Docosahexanoicacid (DHA), mediated by commonly occurring fungi.

Corn is the most widely used raw material in food and feed industry, however, corn is quite low in fats (2.5-7%) and proteins (8-10%).When used as animal feed corn has to be supplemented with soymeal to enrich its protein content[8,4]. The cultivation of yeast, of *Candida spp*[9] *Rhodotorula spp.*[3] can enhance protein, pigments , aroma and good quality fats to the corn based meal.

“Bring back ω-3 fatty acids” envisage enriching human food chain by PUFA includes enriched feed for poultry and dairy animals. Polyunsaturated fatty acids (PUFAs) are essential components of higher eukaryotes and have demonstrated health benefits for animals as well as humans. Currently, production of PUFA by marine microorganisms has been a subject of intense research with increasing commercial attention. Considering the safety and performance aspects, oleaginous marine microbes are the interesting alternative sources to fish oils. Marine yeasts are advantageous for lipid production over other sources, as they can accumulate 20-80% oil of their dry weight and their cultures can be easily scaled up. The typical oily yeast genus includes *Lipomyces, Cryptococcus, Yarrowia, Rhodotorula and Candida*[10]. Optimization of the cultivation media containing wide variety of agro industrial wastes as carbon sources have been studied for biotechnological production of PUFAs[5,11,12]. Reports are available on use of raw materials like corn, cassava starch[8,13,5],pretreated lignocellulosic agricultural wastes like wheat bran, rice bran, corn strover[14,15,] for lipid production by oleaginous yeasts. There are many studies about sourcing PUFA using marine yeasts but most of them deal with fermentation in liquid cultures however solid state fermentation offers greatest possibilities. To our knowledge simultaneous production of both PUFAs and proteins using marine oleaginous yeasts has not been reported yet. Considering these facts in the present study marine yeasts were tested for production of cornmeal based bioproduct using SSF.

## MATERIALS AND METHODS

### *Microorganisms*

Yeasts were isolated from sea water obtained from Konkan coasts of India. Out of 54 isolates, four fast growing yeasts were studied for PUFA production. The yeasts were maintained on the basal media (BM) containing 2g% Dextrose, 1g% yeast extract and sea salt solution. The isolates were identified through microbiological and molecular techniques as, *Rhodotorula muciliginosa (C1), Candida orthopsilopsis (C2), Candida tropicalis (C3), Candida parasilopsis (C4)* and were studied for PUFA accumulation and protein enrichment through SSF on corn based meal.

### *Oleaginous nature of isolates*

The yeasts were cultivated on the basal media for 3 days at 27°C and screened microscopically for lipid accumulation. To detect the lipid bodies wet mount of yeast cells were observed using Leica DCIM 2500 microscope. The intracellular lipid content in yeasts was confirmed by fluorescence microscopy using the Nile red staining method [16](The fluorescence microscope facility was provided by IBB, Univ. of Pune).

### *Effect of substrate*

In the initial experiment, the selected yeasts were grown on BM, incubated at 27°C, at 160 rpm. The biomass was harvested at the interval of 48, 96, 120,148 and 196 h and analyzed for fatty acid composition. In further studies, the BM was supplemented with one of the preselected substrates (g%) like 10% Dextrose, Glycerol 4.0%, malt powder 1%,Flax seed oil 1%, 50% whey, or 10% sodium acetate. Cultures were incubated for 120 hrs. at 27°C., 160 rpm. Biomass harvested was estimated for total protein, fats and fatty acid profile.

### *SSF*

Corn seed meal (10 gm) was sterilized by autoclaving and moistened with sterile 15 ml of BM supplemented with various substrates. The meal thus prepared was inoculated with 1ml of yeast cell suspension (0.25A° at 540nm) and incubated statically at 27°C for 194 hrs. Uniform growth of the yeast was assured by mixing them intermittently. At the end of the experiment the fermented substrate (bioproduct) formed was harvested and dried under controlled heating at 50°C till constant dry weight.

### *Total protein*

The total nitrogen of the bioproduct was estimated using micro-Kjeldhal method. Total protein was calculated using protein factor of 6.38(Indian Standards code 7219-1973).The results were compared with control set that included corn seed powder moistened with BM.

### *Lipid Extraction and Fatty acid analysis*

Lipids from the bioproduct were extracted in Hexane using soxhlet extraction method at 50°C.The solvent was evaporated under Nitrogen gas and total lipids were determined gravimetrically. Fatty Acid methyl Esters (FEMS), were prepared with Sodium methoxide method[17] and analysed by Gas Chromatography.

## RESULTS

### *Microscopy*

Nile red stained yeasts cells (Fig1) were viewed for yellow-gold fluorescence. The yeast isolates exhibit small discrete oil bodies distributed throughout the cytoplasm or a large oil globule depending upon its cell type, when observed as wet mount. The isolates showing 4-8 lipid globules per cell, were selected for further studies.

### *Selection of yeasts*

Supplementation of BM with 10g% Dextrose, Glycerol 2.5g%, Flax seed oil 1g%, 50% whey, or 10g% sodium acetate resulted in increment in C/N ratio and supported biomass formation as well as oil accumulation (data not shown). The yeast named as C2 could accumulate highest amounts of PUFA (54.32%) in medium containing flaxseed oil, followed by dextrose, glycerol, whey and malt powders (table 2).After fermentation, the cornmeal showed increase in protein content irrespective of the culture used(figure 2A). The lipid content of the bioproduct ranged between 3.0 to 10% and was 1-3 times higher than in the substrate (figure 2B). Addition of flax oil and glycerol to corn meal resulted in increased protein and higher PUFA accumulation in the bioproduct (figure 3), except in the case of M4C3 and M5C3 (table 3). The cornmeal supplemented with flax oil (l g%) and glycerol (4.0g%) in SSF produced the bioproducts with PUFA content (50-56 %). After fermentation with *C. tropicalis* the enriched cornmeal showed highest protein content of 18-20%. The bioproduct formed using whey (50%) supplemented cornmeal in SSF accumulated PUFA in the range of 44-48% and 11-18% protein. The essential fatty acids were detected in the bioproduct and the content ranged between 0.71 to 20.22% depending on the additional carbon supplement and the yeast inoculated. The bioproduct resulting from glycerol supplementation of corn meal base contained higher GLA but low total lipid (0.82g%). The corn meal supplementation with 1g% flaxseedoil resulted in GLA production. The corn supplemented with whey resulted in maximal GLA accumulation and the highest GLA was isolated from bioproduct enriched with C1 yeast (17.73%). Highest DHA was detected in the bioproduct enriched with C2 yeast and whey supplement. Additionally Linoleiadic acid was detected in the range of 25-32% in all the fermented bioproducts. Sodium acetate added to the cornmeal based SSF, supported accumulation of all the four important PUFAs i.e. Alpha Linolenic acid, Gamma Linolenicacid, Eicosapentaenoicacid and Docosahexaenoic but no considerable increase in protein content was obtained (Data not shown).

**Table I**

***The protein and oil profile of the selected yeasts grown in Basal media (120hrs).***

| Yeast | Biomass g/l | Protein g% | Oil g/g of dw | Saturated fats % | MUFA% | Omega-6 % | Omega-3% | PUFA% |
|---|---|---|---|---|---|---|---|---|
| C1 | 12.87 | 2.68 | 0.17 | 31.19 | 42.92 | 19.76 | 5.91 | 25.67 |
| C2 | 13.14 | 2.55 | 0.57 | 32.97 | 48.56 | 10.88 | 7.60 | 18.48 |
| C3 | 13.33 | 6.38 | 0.11 | 36.3 | 36.3 | 7.991 | 2.34 | 10.7 |
| C4 | 13.11 | 2.73 | 0.14 | 30.6 | 21.77 | 8.65 | 1.13 | 10.78 |

***(C1:Rhodotorula muciliginosa, C2: C. orthopsilopsis, C3:C. tropicalis, C4: C. parasilopsis)***

**Table II**

***PUFA content of the selected yeasts in BM with different supplements.***

| Yeast | Glucose10g% (M1) | Maltpowder 1.0g% (M2) | Glycerol 4.0g%, (M-3) | Whey50%v/v (M-4) | Flaxoil 1.0g% (M-5) |
|---|---|---|---|---|---|
| C1 | 09.48 | 10.29 | 33.92 | 24.43 | 21.11 |
| C2 | 48.339 | 31.412 | 42.76 | 32.86 | 54.32 |
| C3 | 13.127 | 10.93 | 33.96 | 19.22 | 32.22 |
| C4 | 14.40 | 10.97 | 24.33 | 29.64 | 27.24 |

**Table III**

***Lipid production by marine yeasts cultivated as SSF on cornmeal based substrates. (%area of fatty acids in GC analysis)***

| Culture | C18:3n3 | C18:3n6 | C20:5n3 | C22:6n3 | PUFA %area | LCPUFA/Oil mg/gm |
|---|---|---|---|---|---|---|
| MC1 | .22 | 1.01 | .323 | 00 | 45.76 | 33.9 |
| MC2 | .22 | 1.32 | 0.30 | 00 | 50.94 | 36.8 |
| MC3 | .25 | 1.09 | .248 | .345 | 49.21 | 41.2 |
| MC4 | .21 | 1.00. | .187 | Nd | 43.11 | 25.8 |
| M1C1 | .27 | .466 | .363 | Nd | 30.24 | 21.8 |
| M1C2 | .25 | .704 | .382 | 00 | 44.12 | 26.4 |
| M1C3 | .22 | .49 | .198 | .23 | 41.13 | 11.8 |
| M1C4 | .19 | 1.01 | .181 | 00 | 46.95 | 27.8 |
| M2C1 | .18 | 1.215 | .2676 | 00 | 49.01 | 32.2 |
| M2C2 | .22 | .628 | .324 | Nd | 44.7 | 22.6 |
| M2C3 | .19 | .932 | .2580 | Nd | 46.17 | 26.6 |
| M2C4 | .19 | 1.161 | .258 | Nd | 48.23 | 32.4 |
| M3C1 | .14 | .467 | .382 | .021 | 22.95 | 19.4 |
| M3C2 | .21 | .885 | .324 | 00 | 47.43 | 28.2 |
| M3C3 | .21 | 1.00 | .267 | .019 | 47.89 | 31.4 |
| M3C4 | .19 | .875 | .2771 | .028 | 46.76 | 27.4 |
| *M4C1* | *.19* | *17.73* | *Nd* | *2.29* | *48.1* | *404.4* |
| M4C2 | .44 | 4.66 | Nd | 4.9 | 38.87 | 200 |
| M4C3 | .17 | 2.95 | .105 | 1.93 | 47.07 | 103.12 |
| M4C4 | .40 | 2.589 | .544 | .4 | 58.3 | 78.6 |
| M5C1 | .53 | Nd | .27 | Nd | 50.0 | 16.6 |
| M5C2 | .64 | Nd | .33 | Nd | 56.0 | 19.4 |
| M5C3 | .47 | ARA2.66 | .31 | .03 | 54.0 | 67.0 |
| M5C4 | .43 | Nd | .28 | Nd | 53.0 | 14.2 |
| M6C1 | 1.4 | 1.23 | Nd | .59 | 43.0 | 64.6 |
| M6C2 | Nd | 1.29 | Nd | .956 | 39.0 | 44.8 |
| M6C3 | Nd | 1.2 | Nd | .87 | 34.0 | 25.4 |
| M6C4 | Nd | 1.03 | Nd | 1.03 | 43.0 | 41.2 |

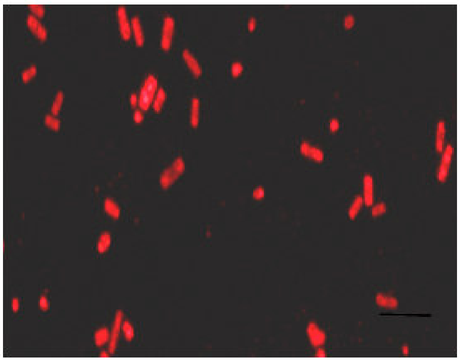
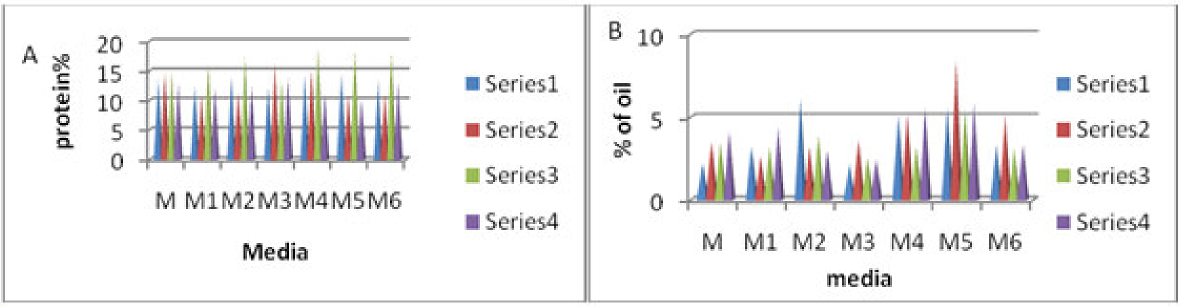


**Figure I**

*Flourescence staining of marine  oleaginous yeast (Candida tropicalis).Bar indicates 10um*

**Figure II**

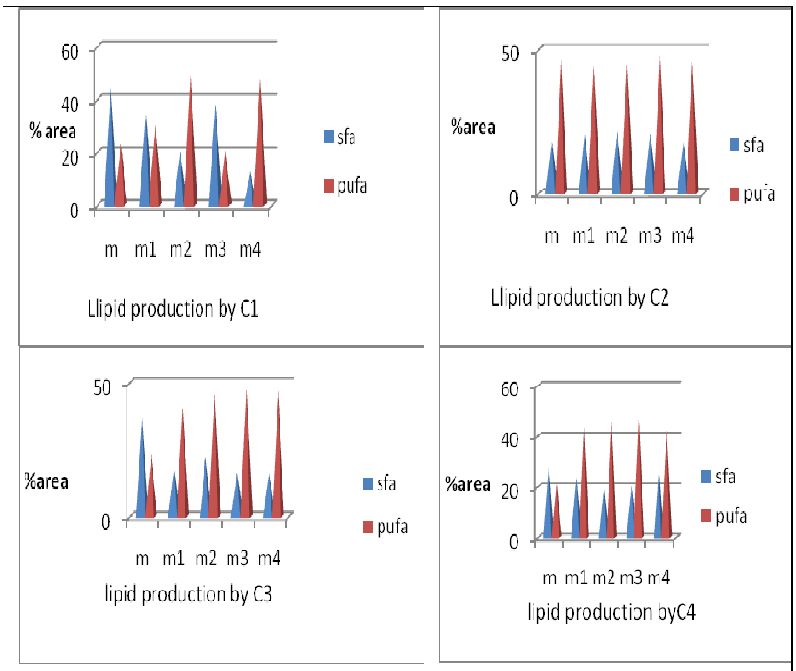
*Effect of substrates on Protein (A), oil(B) content of various  yeasts grown on cornmealbase.*




*(M:cornpowder,M1:M+10%Glucose,M2:M+maltpowder,M3:M+GlycerolM4:M+Whey,M5:M+Flaxoil,M6:M+sodium acetate., Series 1:isolate C1,  series 2:isolate C2, series 3: isolate C3,  series 4:isolate C4 as estimated By micro Kjheldal method and gravimetrically, respectively.*

**Figure III**

*Effect of various supplements on lipid accumulation by marine yeasts (isolate code:C1,C2,C3, C4) as estimated by GC.*



## DISCUSSION

A number of factors have been found to affect both the fatty acid compositions and the percentage of lipid bodies found in the fungi, which imply that the lipid composition of the moulds can be manipulated in order to obtain the fatty acids of interest.[18] Four yeasts of marine origin were tested for their ability to grow and enrich corn powder with PUFA and protein. These yeasts used in the present study were chosen according to their capacity to synthesize PUFA (Table1) in the preliminary study that was performed with 14 strains under submerged cultivation (data not shown). Various amounts of dextrose, flax oil, whey, glycerol, sodium acetate, malt powder were added to the basal media. The supplementation to the liquid basal media was based on the hypothesis that these additions may stimulate yeast growth, and allow the organism to accumulate higher amounts of unsaturated fatty acid. The potential of the marine isolates to transform corn powder (meal) supplemented with various carbon sources into desired bioproduct was tested in SSF.

SSF: Cereal grains such as maize, wheat and barley, are the main source of dietary energy in animal nutrition. Maize is also an excellent source of linoleic acid. The seed is high in starch and supports microbial growth. The growth of the microorganisms requires the assimilation of the polysaccharides contained in the cornmeal. According to Leach *et al.*[13], the starches, the sterilization leads to a more or less complete solublization of the com starch. This soluble form of the starch is available for the growth of the yeasts in the same manner as Merck soluble starch. Corn is a well-balanced source of assailable carbon with adequate levels of organic nitrogen and other nutrients necessary for fungal proliferation. The total lipid quantity of yellow corn, the substrate was 2.5g% and the linoleic acid was 52.3% as detected by GC analysis. From the view point to enrich the yellow corn with essential fatty acids and increase the total PUFA content, SSF experiment was conducted. All the studied yeasts produced nearly13% g/L of biomass but differ in the concentration of protein, total lipids and PUFA accumulated. The variations in metabolite accumulation could be correlated with cell morphology and physiology. The maximum protein content was detected in C3 (6.3%) and the highest PUFA accumulation was observed in C1 (25.67%) grown on BM for 120 hrs. at 27°C.The addition of dextrose, glycerol, whey and flax was found to be beneficial and resulted in increased accumulation of PUFA (Table2).The yeasts were cultivated in SSF using corn meal supplements for 196 h. Initial lipid, protein content of the corn meal with and without cultivated yeast was detected. Considering the initial nutritional composition of the corn meal, it could be said that fermentation by marine yeasts resulted in nutritional enhancement of the corn meal. Most of the oleaginous yeasts accumulate storage lipids when a nutrient (commonly Nitrogen) becomes exhausted and carbon source is still available and continues to be assimilated by the cells which progressively become obese.[19] Efficiency of the carbon utilization and its biotransformation to various metabolites depends on carbon availability from cereal materials. Although cereals provide both sufficient carbon contents in the form of starch and suitable C/N ratio ranging commonly from 20 to 60 various amounts of either glucose, maltpowder, whey, flaxoil, glycerol were added to the cereal substrate to test their effect on PUFA and protein biosynthesis. Addition of 10g% Glucose to BM caused increased accumulation of PUFA in all the yeasts except in pigmented yeast *Rhodotorula.* However, SSF of the cornmeal substrate with and without Glucose (10g%) supplement yielded the bioproduct with 41-50% PUFA content. As per the reported data [20], glycerol is found to be effectively utilized by various yeasts and produced high biomass and lipids specially, TAGs (Triacylglycerol).[21] bioconversion of glycerol using oleaginous yeast *Rhodosporidium toruloides,* reported lipid yield of 17.5 g/100 g consumed glycerol and the predominant fatty acids were palmitic acid, stearic acid, oleic acid, and linoleic acid. [21] In the present study the fatty acid composition of the yeasts growing in SSF showed decrease in saturated faty acids(SFA) (total saturated fats 16.3% to 20.9%) and high PUFA content (Table.3) Lower filamentous fungi possessing active oil-biotransforming system are able to utilize exogenous oils containing individual fatty acid precursors and convert them to PUFAs [22]. In the present study the ability of the yeasts to utilize flax oil and convert fatty acid precursors in to PUFA in SSF system was studied. The results clearly illustrate the increase in the yield of the total lipid and PUFA content due to fermentation of flax oil (1g%) supplemented cornmeal by marine yeasts. Flaxoil from the substrate was utilized by the yeasts and converted to microbial PUFA and interestingly, SFA decreased in all bioproducts. The bioproduct analysis showed an increase of PUFA with accumulation of important PUFAs ALA GLA EPA DHA (Table3). As mentioned by Aggelis[24] that either fatty acids are degraded for growth requirements or used as a substrates for the endocellular biotransformation process, leading to concentration changes and production of 'new' fatty acids that were not previously present in the substrate. Similar results are reported in solid state fermentation of Mucor grown on agro industrial wastes. Whey is a potential raw material and has been used on large scale for SCP production [24,25] that can be used as livestock feed. Also whey is considered as a good substrate for production of PUFA and pigments in submerged media.[26] Cheese whey as renewable source for microbial lipid production using zygomycetes.[27] The production of SCP and SCO rich in saturated fatty acids in shake flasks is reported from cheese whey using kefir microorganisms like *K. fragilis, C.curvurata* etc.[28,29,26] In the present study the shake flask experiment using whey (50% v/v) resulted in higher yield of PUFA All the *Candida* isolates C2, C3, C4 showed 43.75%, 44.32% and 63.63% rise in PUFA accumulation respectively when grown on M4(BM with 50% whey) in submerged condition. In SSF studies, corn supplemented with whey (50%v/v) resulted in accumulation of high protein as well as PUFA and enriched the bioproduct. The cultivation of a yeast such as *Candida tropicalis* on corn meal leads directly to a balanced feed. The cornmeal after fermentation with *C. tropicalis* was enriched with 15 to 20% protein, these observations are similar to earlier reports.[9] The resultant increase in protein content of the bioproduct is due to the presence of the synthesized yeast biomass. The bioproduct formed using whey (50%) supplemented cornmeal in SSF accumulated 11-18% protein and PUFA in the range of 44-48%. The fermented corn meal enriched by SCP and PUFA could be used as animal feed supplement. The application of whey as substrate supplement for enhancement of nutritional quality of cornmeal by SSF using marine yeasts represents a promising process for feed production at low cost. *Rhodotorula* mediated fermentation adds carotenoid pigments to the bioproduct (data not shown).

## CONCLUSION

The study provides a simple method of SSF of corn powder using marine oleaginous yeasts. Our results indicate that SSF offers favorable cultivation environment for simultaneous enrichment of corn powder with PUFA and proteins using marine oleaginous yeasts. This value added bioproduct may be used as low cost feed supplement and may create new perspectives for PUFA and protein rich cereal based balanced feed production.

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