Original article

Antifungal activity of biopolymers containing natamycin and rosemary extract against *Aspergillus niger* and *Penicillium roquefortii*

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Summary Antimicrobial agent-releasing films have been proposed as an effective way of inhibiting chiefly surface spoilage of food products. Antifungal activities of natamycin (NA), rosemary extract (RE) and NA + RE were tested against *Aspergillus niger* and *Penicillium roquefortii* with agar disc diffusion assay. NA, RE and NA + RE were also included into biopolymers made from gluten and methyl cellulose. Minimum inhibitory concentrations (MIC) of NA in both films were 2 and 1 mg NA per 10 g film solution against *A. niger* and *P. roquefortii*, respectively. RE did not show any inhibitory antifungal activity alone. Although NA incorporated into both films at a concentration of 1.5 mg NA per 10 g film solution was not effective against *A. niger*, combination of NA at the same concentration with RE in the films inhibited the growth of this mould. NA in solution or in biopolymers is very effective in inhibiting the growth of selected organisms, and RE acted synergistically with NA to prevent the growth of *A. niger* when incorporated into both films.

Keywords Antimicrobial polymer, methyl cellulose film, natamycin, rosemary extract, wheat gluten film.

Introduction

Microbial growth on food surfaces, which could originate from post-processing contamination during handling and packaging leads to food spoilage, serious illnesses and consequent losses for the food industry. Antimicrobial agents could be used on the food surfaces via direct application techniques, such as dipping, spraying or brushing to defuse contamination. Direct application of antimicrobial substances may result in neutralisation or evaporation of active agents and rapid diffusion from surface to interior parts of foods (Coma *et al*., 2002). Incorporation of natural antimicrobial agents into bio-based packaging materials is an innovative way of overcoming these problems. Functional bio-based packaging materials could not only extend the shelf-life of the food products, but could also eliminate environmental concerns resulting from utilisation of synthetic polymers (Cha & Chinnan, 2004).

Biopolymers prepared especially from protein and polysaccharide materials, such as soy protein, corn zein and alginate films, have been studied as carriers for numerous antimicrobial agents (Padgett *et al*., 1998, 2000; Cutter *et al*., 2001; Li *et al*., 2006). Organic acids, lysozyme, potassium sorbate and several plant extracts and oils were included into biopolymers owing to their antimicrobial properties (Quattara *et al*., 2000; Ozdemir & Floros, 2003; Min *et al*., 2005; Pranoto *et al*., 2005; Seydim & Sarikus, 2006).

Biopolymers having antimicrobial activity were generally prepared by incorporating one type of active agent into the film. However, it has been known that some antimicrobial agents might have synergistic effects in inhibiting bacterial growth when they are used in combination (Nazer *et al*., 2005). There are several examples of the use of combinations of antimicrobials in packaging. Garlic oil, potassium sorbate or nisin was added to chitosan to enhance its antimicrobial activity, and it was concluded that the antimicrobial activity of chitosan was improved with the addition of garlic oil without causing any significant effect on the physical and mechanical properties of the film (Pranoto *et al*., 2005). In another study, it was reported that grape seed and green tea extracts enhanced the antimicrobial activity of nisin against *Listeria monocytogenes* in soy protein coating (Theivendran *et al*., 2006). Examples in the scientific papers reveal that combinations of

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antimicrobial agents incorporated into packaging films could act synergistically; therefore, they could have improved effectiveness against microorganisms. Hence, further researches in synergistic interaction of antimicrobial agents are promising.

Natamycin (NA) is a natural antifungal agent produced by *Streptomyces natelensis*. It inhibits the fungi by binding to cell membrane sterols, especially ergesterol; therefore, membrane permeability is enhanced, resulting in lyses. NA was approved as a generally recognised as safe (GRAS 21 CFR 172.155, FDA-ASP/1577, 007681-93-8) agent by Food and Drug Administration (FDA) in the United States, and was assigned as a natural preservative in European Union (EEC no. 235). Its effectiveness in preventing the mould growth on cheese and sausage has been reported (Stark, 2003). Although NA-incorporated casein coating was not very effective in inhibiting mould growth on Kashar cheese during ripening (Yildirim *et al*., 2006), cellulose-based film containing NA inhibited the growth of *Penicillium roquefortii* on the surface of Gorgonzola cheese (de Oliveira *et al*., 2007).

Antimicrobial and antioxidant properties of plant extracts make them valuable alternatives in preservation of food products (Schwarz *et al*., 2001; Rey *et al*., 2005). Rosemary (*Rosmarinus officinalis*) is a plant which is native to Mediterranean region. Several scientific studies investigated the antimicrobial and antioxidant properties of *Rosmarinus officinalis* species and their main constituents (Pandit & Shelef, 1994; Basaga *et al*., 1997; Del Campo *et al*., 2000; Celiktas *et al*., 2007). It was reported that rosemary extracts (RE) and essential oils have strong antioxidant activity (McCarthy *et al*., 2001). While RE was effective against gram-positive bacteria, such as *Leuconostoc mesenteroides*, *Listeria monocytogenes* and *Staphylococcus aureus*, it had no activity on gram-negative bacteria (Del Campo *et al*., 2000). Antioxidant and antimicrobial properties of rosemary are attributed to phenolic diterpenes, such as carnosic acid, carnosol, rosmanol, epirosmanol, isorosmanol, methyl carnosate and other phenolic acids, such as rosmarinic acid (Schwarz & Ternes, 1992; Cuvelier *et al*., 1996).

The aim of this study is to evaluate and compare the antifungal activities of paper discs and wheat gluten (WG) and methyl cellulose (MC)-packaging films containing NA, RE and NA + RE combination against *Aspergillus niger* and *P. roquefortii*.

Materials and methods

Natamycin source and rosemary extraction

Pimalac® used as NA source was provided by Mayasan (Istanbul, Turkey). RE was produced using a modified procedure of Madsen *et al*. (1998). Rosemary leaves were collected from their natural habitat, and extraction was performed immediately. Leaves (12 g) were homogenised in 70 mL absolute ethanol at 26 000 r.p.m. (Heidolph Silent Crusher M Homogenizer, Germany) for 5 min. The solution obtained was stirred for 30 min in dark and cenrifugated at 5000 r.p.m. (Nuve NF 615, Turkey) for 5 min. Supernatant was collected and 30 mL ethanol was added to precipitate, and same procedure was repeated twice. Twenty millilitres of ethanol was used in the last extraction step. Collected supernatant was evaporated under vacuum at 40 °C for approximately 1 h with a rotary evaporator (Heidolph Laborato4000, Germany). The extract obtained was vacuum filtered through 5-μm cellulose nitrate filter.

Preparation of fungal cultures

A. niger was isolated from onion skin in Plant Protection Department of Mustafa Kemal University (Hatay, Turkey), and *P. roquefortii* DBCI-1 was isolated from Danish blue cheese in Food Engineering Department of Izmir Institute of Technology (Izmir, Turkey). Identity of both fungi was confirmed with microscopy and morphological analysis. All fungi were grown on potato dextrose agar (PDA) (Merck, Darmstadt, Germany). Fungi were maintained on PDA slants at 30 °C to ensure viability and purity. Spores were collected with sterile 0.1% peptone water from 3- to 5-day-old subcultures for *A. niger* and 5- to 7-day-old cultures for *P. roquefortii*. To prevent contamination, cultures were refreshed from stock solutions in glycerol every 2 weeks. Homogenised culture solutions were transferred to sterile tubes. Spore count was determined by spreading culture on Thoma slide and counting under microscope. Necessary dilutions were made with 0.1% peptone water to obtain 10^4 and 10^6 spore per mL counts for *A. niger* and *P. roquefortii*, respectively. Hundred microlitres spore solution was spread onto PDA agar plate (Sebti *et al*., 2005).

Preparation of wheat gluten and methyl cellulose films and incorporation of active agents

WG films were prepared according to a method by Pochat-Bohatier *et al*. (2006) with some modifications. Fifteen grams of WG (Sigma-Aldrich, Germany) was dissolved in 31.5 mL absolute ethanol with mixing. Then, 0.03 g sodium sulfite, 3 g glycerol and 63 mL distilled water were added to the solution and homogenised. The pH of film solution was set to 4 with acetic acid, and the solution was mixed and heated to 70 °C on a magnetic stirrer. Ten grams of film solution (fs) was spread onto 8.5 cm diameter polystyrene Petri dishes and dried at 30 °C.

A procedure by Turhan & Sahbaz (2004) with some modifications was used for the preparation of MC films.

3 g MC (Sigma-Aldrich, Germany) was mixed with 50 mL distilled water. 50 mL ethanol was added and homogenised. After addition of 1 mL glycerol, solution was heated to 80 °C. 10 g of fs was spread onto polystyrene Petri dishes and dried at 30 °C.

The NA-containing films were prepared exactly like control films, but NA in powder form was added to films just before spreading fs onto Petri dishes at a temperature of 50–55 °C. After addition of NA, fs was mixed for about 5 min with a magnetic stirrer. Concentration of NA was in the range of 0.2–40 mg per 10 g fs.

RE-containing films were obtained by replacing a certain volume of ethanol and water in film formulation with RE (2.3 mL water and 0.7 mL alcohol for 3 mL RE in 10 g fs). NA + RE-containing films were prepared according to procedures explained earlier and adding RE and NA before spreading fs onto plate. The average thickness of films (mm) was measured randomly at 10 points with a hand micrometer (SHAN Electronic, China). The average thicknesses of WG films ranged between 0.23 to 0.25 mm, and there was no statistical difference between thickness values of control and active agent added films. MC films had average thicknesses of 0.63–0.69 mm. MC film containing 20 mg NA per 10 g fs (0.75 mm) was the only significantly different one in terms of thickness.

Agar disc diffusion assay

NA was dissolved in glycerol/ethanol (1:1, v/v) solution. Filter paper and films were cut into 1×1 cm squares. Twenty microlitres of NA of 100–5000 ppm, RE as prepared and NA + RE combination (25–2500 ppm NA + 50% RE) were impregnated in square discs. Discs were placed on PDA agar plates seeded with either 10^4 spores per mL *A. niger* or 10^6 spores per mL *P. roquefortii*. *A. niger*-containing plates were incubated for 3 days at 30 °C, while incubation time for plates with *P. roquefortii* was 6 days at 30 °C. Controls were also prepared by absorbing distilled water, peptone water, glycerol, ethanol and glycerol + ethanol. Inhibition zones were measured with a micrometer (Espinel-Ingroff *et al.*, 2007).

Same procedure was also applied to films. Instead of paper discs, 1 cm^2 films were used. WG or MC films, which did not contain any active agent, were used as the controls. At least three replicates of each treatment were performed. Replicates were prepared using different batches of active agents.

Statistical analysis

Data were analysed by analysis of variance (ANOVA) using MINITAB (version 14.10). Means were compared using Fisher least significant difference (LSD) method at $P = 0.05$.

Results and discussion

Antifungal activities of paper discs

In the first part of this study, antifungal activities of NA, RE and their combinations were tested against *A. niger* and *P. roquefortii*, which are prevalent spoilage organisms in dairy products. Antifungal activity was determined by measuring the zones formed around paper discs or films containing active agents or controls. Diameter of discs or films was also included in inhibitory zone diameter. Table 1 shows the effect of NA concentration on growth inhibition of *A. niger* and *P. roquefortii*. NA containing paper discs demonstrated inhibition zones ranging from 1.5 to 3.97 cm at the concentration range of 750–5000 ppm against *A. niger*, and 3.02–5.32 cm for *P. roquefortii*. Minimum inhibitory concentration (MIC) values were chosen as the boundary between no effect and inhibitory effect against tested fungus. The MIC value of NA for *A. niger* and *P. roquefortii* is 750 ppm. These values are higher than reported by Stark (2003). Contrary to agar disc diffusion assay used in our study, antifungal activity was tested directly spreading a certain volume of NA suspension on agar in the previously mentioned study. In addition, commercial NA that was used in this study contains 50% NA and 50% lactose. Lactose does not have antimicrobial activity itself, but rather is an energy source for fungus. Therefore, it could enhance the growth of tested micro-organisms. Despite this, NA was very effective in inhibiting the growth of *A. niger* and *P. roquefortii*. Stark (2003) also reported that 0.01–0.02 mg cm^{-2} NA was effective against the moulds during surface application. In terms of surface area,

Table 1 Inhibition zone diameters of *Aspergillus niger* and *Penicillum roquefortii* exposed to natamycin (NA) absorbed onto paper discs at different concentrations in agar disc diffusion assay

NA conc. [ppm (mg cm^{-2})]	Diameter of inhibition zone (cm)	
	*A. niger**	*P. roquefortii*†
0 (0)	+	+
250 (0.005)	+	+
500 (0.01)	+	+
750 (0.015)	1.5 ± 0.0[a]	3.02 ± 0.11[a]
1000 (0.02)	1.66 ± 0.12[a]	3.18 ± 0.24[a]
1250 (0.025)	2.62 ± 0.27[b]	4.17 ± 0.72[b]
2500 (0.05)	3.49 ± 0.32[c]	4.30 ± 0.24[b]
5000 (0.1)	3.98 ± 0.3[d]	5.32 ± 0.43[c]

+ No inhibition; [a–d] same letters show that there is no statistical difference between different NA levels at $P > 0.05$.
*Measurements were recorded after 3 days of incubation at 30 °C.
†Measurements were recorded after 6 days of incubation at 30 °C.

Table 2 Inhibition zone diameters of *Aspergillus niger* and *Penicillum roquefortii* exposed to natamycin (NA), rosemary extract (RE) and NA + RE-containing wheat gluten (WG) films in agar disc diffusion assay

NA conc. (mg per 10 g fs)	RE conc. (mL per 10 g fs)	Diameter of inhibition zone (cm) *A. niger**	*P. roquefortii*†
0	0	+	+
0.5	0	+	+
1	0	+	2.34 ± 0.15[a]
1.5	0	+	2.72 ± 0.08[b]
2	0	2.76 ± 0.4[a]	3.11 ± 0.11[c]
5	0	3.72 ± 0.18[b]	4.46 ± 0.38[d]
10	0	3.98 ± 0.06[c]	5.23 ± 0.22[e]
20	0	4.01 ± 0.0[c]	5.26 ± 0.20[e]
40	0	4 ± 0.09[c]	5.06 ± 0.11[f]
0.5	1.5	+	+
1	1.5	+	2.5750 ± 0.49[a]
1.5	1.5	1.79 ± 0.11[a]	2.7750 ± 0.21[b]
2	1.5	2.53 ± 0.09[b]	3.2833 ± 0.04[c]
5	1.5	3.58 ± 0.07[c]	4.1583 ± 0.01[d]
10	1.5	3.74 ± 0.11[d]	4.8250 ± 0.01[e]
20	1.5	4.08 ± 0.07[e]	5.4167 ± 0.07[f]
0.5	3	+	+
1	3	+	2.15 ± 0.13[a]
1.5	3	1.71 ± 0.06	2.65 ± 0.18[b]

+ No inhibition; [a–f] Same letters show that there is no statistical difference between different levels at $P > 0.05$; fs, film solution.
*Measurements were recorded after 3 days of incubation at 30 °C.
†Measurements were recorded after 6 days of incubation at 30 °C.

MIC values of NA against *A. niger* and *P. roquefortii* in our study were found as 0.015 and 0.0175 mg cm^{-2}, respectively. These values are in the range of that reported by Stark (2003). MIC value of NA for *P. roquefortii* was the same for *A. niger*, although NA was tested against a higher spore load in case of *P. roquefortii*. With increasing NA concentration, diameter of inhibition zone also increased (Table 1).

While NA was very effective against both fungi, even 100% RE did not show any inhibitory action against *A. niger* and *P. roquefortii*. Contrary to our results, it was reported that commercial RE dissolved in ethanol slowed the growth of *P. roquefortii*, and antibacterial activity was strongly influenced by media composition, such as pH and salt content (Del Campo *et al.*, 2000). In the previous studies in literature, various procedures were used for the extraction of rosemary, and antimicrobial activity showed variations depending on the extraction method and solvent. In a study, it was found that methanolic extracts of rosemary had low antimicrobial activities (Celiktas *et al.*, 2007). According to another research, while supercritical fluid extract of rosemary had a strong antioxidant activity, its antimycobacterial activity was low (Leal *et al.*, 2003).

There was no change in MIC values when NA was combined with RE in paper disc form (data is not shown). Therefore, synergy was not observed in this case.

Antifungal activities of biopolymers

NA, RE and their combinations were also incorporated into WG and MC films, and their antifungal properties were tested. Tables 2 and 3 list the diameter of fungal inhibition zone for WG and MC films containing various concentrations of NA and NA + RE combinations. Examples of inhibition zones formed around biopolymers are also shown in Figs 1 and 2. NA-added films were very effective in inhibiting the growth of both micro-organisms. The MIC values of NA were 2 mg per 10 g fs for WG and MC films against *A. niger* and were 1 mg per 10 g fs against *P. roquefortii*. As NA concen-

Table 3 Inhibition zone diameters of *Aspergillus niger* and *Penicillum roquefortii* exposed to natamycin (NA), rosemary extract (RE) and NA + RE-containing methyl cellulose (MC) films in agar disc diffusion assay

NA conc. (mg per 10 g fs)	RE conc. (mL per 10 g fs)	Diameter of inhibition zone (cm) *A. niger**	*P. roquefortii*†
0	0	+	+
0.5	0	+	+
1	0	+	2.59 ± 0.09[a]
1.5	0	+	3.04 ± 0.13[b]
2	0	2.84 ± 0.18[a]	3.37 ± 0.11[c]
5	0	3.51 ± 0.04[b]	4.54 ± 0.35[d]
10	0	3.64 ± 0.2[c]	4.91 ± 0.11[e]
20	0	4 ± 0[d]	4.89 ± 0.13[e]
40	0	3.99 ± 0.11[d]	5.30 ± 0.17[f]
0.5	1.5	+	+
1	1.5	+	2.38 ± 0.14[a]
1.5	1.5	2.29 ± 0.34[a]	2.77 ± 0.27[b]
2	1.5	2.75 ± 0.21[b]	2.93 ± 0.08[b]
5	1.5	3.63 ± 0.14[c]	4.52 ± 0.14[c]
10	1.5	4.09 ± 0.06[d]	5.10 ± 0.19[d]
20	1.5	4.2 ± 0.11[d]	5.46 ± 0.08[e]
0.5	3	+	+
1	3	+	2.39 ± 0.10[a]
1.5	3	1.88 ± 0.05	3.04 ± 0.12[b]

fs, film solution; + no inhibition; [a–h] same letters show that there is no statistical difference between different levels at $P > 0.05$.
*Measurements were recorded after 3 days of incubation at 30 °C.
†Measurements were recorded after 6 days of incubation at 30 °C.

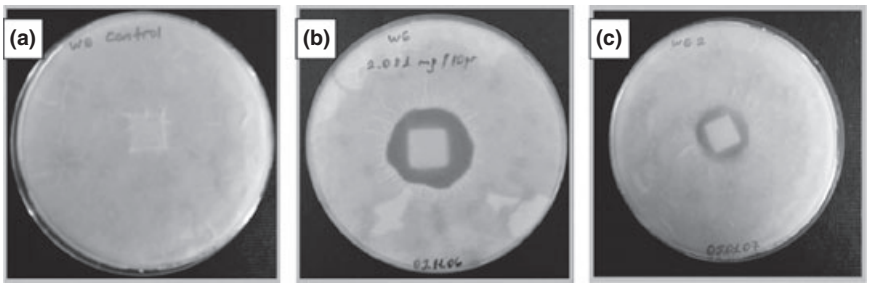

Figure 1 Pictures showing inhibition zones of *Aspergillus niger* exposed to wheat gluten films: (a) control; (b) 2 mg NA per 10 g film solution (fs); (c) [2 mg NA + 1.5 mL rosemary extract (RE)] per 10 g fs.

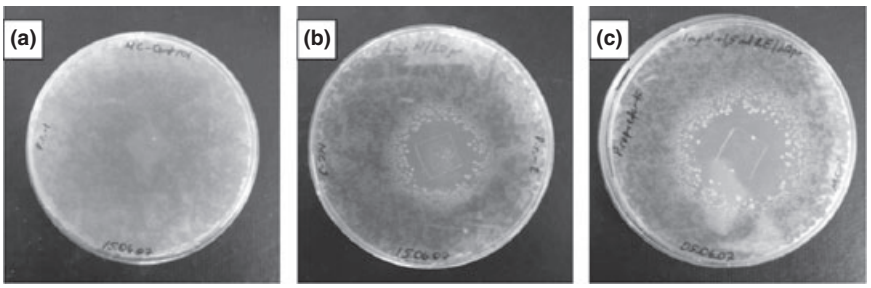

Figure 2 Pictures showing inhibition zones of *Penicillium roquefortii* exposed to methyl cellulose films: (a) control; (b) 2 mg NA per 10 g film solution (fs); (c) [2 mg NA + 1.5 mL rosemary extract (RE)] per 10 g fs.

tration increased from 2 to 40 mg per 10 g fs, the inhibition zone diameter also increased from 2.83 to 4 cm for MC film and 2.76 to 4 cm for WG films on agar medium for *A. niger* (Tables 2 and 3). Inhibition zone diameters of *P. roquefortii* varied between 2.34 and 5.06 cm and 2.59 and 5.30 cm for WG and MC films, respectively. The NA inhibition zone diameters were very close and the NA MIC values were the same for WG and MC films. Although MC is a carbohydrate and WG is a protein-based film, the structure of the film did not affect the release behaviour of NA from the films. There was a leveling out at inhibition zone diameters around 10 mg NA per 10 g fs meaning that adding more than this level of NA is unnecessary. Pranoto *et al.* (2005) also reported that increasing the level of nisin and garlic oil at higher concentrations did not contribute to the inhibitory effect of chitosan film owing to the maximum capability of chitosan polymer.

When it is desired to compare the MIC values for paper discs and films, it is more convenient to use MIC values in terms mg cm^{-2} (Table 4). The MIC values of NA against *A. niger* for both films (0.035 mg cm^{-2}) are more than twice the MIC value for paper discs (0.015 mg cm^{-2}). Antimicrobial agents were absorbed on paper discs while they were added into film matrix during preparation of biopolymers. Therefore, release mechanism of active agents from discs and film might differ. As antimicrobial agents are in the film matrix interaction of active agents with film compounds could result in a decrease in antimicrobial activity in the films. Although NA MIC value against *P. roquefortii* in film form was higher (0.0175 mg cm^{-2}) compared with paper discs (0.015 mg cm^{-2}), the difference was much lower. This is probably related to the susceptibility of *P. roquefortii* to NA as reflected in MIC value also.

The WG and MC films containing RE alone did not have any antifungal activity. However, RE had a synergistic effect on NA in both films. Although the MIC value of NA for WG and MC films was 2 mg NA per 10 g fs for *A. niger*, zones formed at 1.5 mg NA per

Table 4 Minimum inhibitory concentration (MIC) values of natamycin (NA), rosemary extract (RE) and NA + RE in paper disc and film form against *Aspergillus niger* and *Penicillum roquefortii*

	Disc		WG film		MC film	
Fungus	**NA***	**NA + RE**	**NA†**	**NA + RE‡**	**NA†**	**NA + RE‡**
A. niger	750 (0.015)	ns	2 (0.035)	1.5 + 1.5 (0.0264 + 0.0264)	2 (0.035)	1.5 + 1.5 (0.0264 + 0.0264)
P. roquefortii	750 (0.015)	ns	1 (0.0175)	ns	1 (0.0175)	ns

WG, wheat gluten; MC, methyl cellulose; ns, no synergy.
*ppm (mg cm^{-2}).
†mg per 10 g film solution (fs) (mg cm^{-2}).
‡mg NA per 10 g fs + mL RE per 10 g fs (mg NA per cm^2 + mL RE per cm^2).

10 g fs when 1.5 mL RE per 10 g fs were also added to films (Table 2). However, there were no significant changes in the zone diameters at higher NA concentrations as compared with films containing only NA. Therefore, the effect of RE was only apparent at 1.5 mg NA per 10 g fs for *A. niger*. Furthermore, increasing the RE amount to 3 mL did not decrease MIC values and did not increase the zone diameters further for *A. niger*. Synergistic interaction was not observed between NA and RE for *P. roquefortii* (Table 3).

Conclusions

This study showed that NA is a very effective antifungal agent when incorporated into biopolymers of WG and MC. Although RE alone does not inhibit fungal growth, it could decrease the MIC value of NA. Developed films might be used to prevent the spoilage of dairy products caused by *A. niger* and *P. roquefortii*. However, these *in vitro* studies need to be supported with real application studies.

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