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The impact of various environmental and dietary variables on production of pectinase biosynthesis by *Aspergillus niger*.

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ABSTRACT : ***Pectinase enzymes are commercially important enzyme with a wide range of applications, particularly in food industry. Pectinases accounts for 25 % of all food enzymes produced worldwide, and their market is growing by day. The main objective of this study was to isolate and investigate the most producer microorganisms for pectinase enzyme from different sources. Out of fifty tested isolates, isolate no. (2) from Zagazig, Belbeis city, was the most potent that produce pectinase enzyme, and identified as Aspergillus niger. After that maximizing pectinase production by optimization the cultural conditions as incubation temperatures, incubation periods, pH, static and shaking conditions and nutritional conditions carbon sources, nitrogen sources and phosphorus sources. The highest production of pectinase enzyme by local isolate Aspergillus niger was obtained after 4 days of incubation at 30ºC and medium was adjusted to pH 6, supplemented with addition of yeast (0.8 g/100 mL), sucrose (0.6 g/100 mL) and Na2HPO4.***

KEYWORDS Pectinase; Aspergillus niger; Cultural conditions; Enzyme activity

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I. INTRODUCTION

Microbial enzymes have a wide range of technological applications in many industrial processes. Among the many enzymes that have been marketed, there are many products of fermentation of filamentous fungi [1].Pectic materials are polysaccharides with complicated structural properties found in the middle lamella and primary cell wall of higher plants [2]. Pectin is colloidal polysaccharides, with galacturonic acid backbone linked by α (1-4) linkage [3]. Because of their key function in the plant-pathogen interaction, enzymes that breakdown the plant cell wall (PCW) have been intensively researched. However, because of their well-documented biotechnological potential, the synthesis, characteristics, and uses of these enzymes have sparked a lot of interest [4].

Aim of the study was interested for maximization the production of pectinase enzyme by environmental and nutritional requirements from local fungal isolates.

II. MATERIALS AND METHODS

Collection of Samples

Fungi examined for their pectinase activity in the current study were isolated from various ripe fruits and soil samples. Apples and oranges were among the ripe fruit samples collected. In addition, soil samples were taken from several locations in Sharkia and Qalyobia Governorate, Egypt. All samples were collected aseptically in sterile bags and transported to the Microbiology Research Laboratory at Botany and Microbiology Department, Faculty of Science – Zagazig University during year 2018 to 2019.

Isolation and screening of pectinase producing fungi

According to the protocol of [**5]**, the samples were collected with slight modifications. Ten g air dried soil sample were suspended in 100 ml sterile saline solution (0.85% NaCl in distilled water) in a liter Erlenmeyer conical flasks, shaken for 15 min, then left for 30 min to sediment the soil particles 10 ml of the supernatant were taken and added to 90 ml sterile saline solution. After shaking for 10 min, subsequent dilutions were made by the same manner. 1 ml of each dilution was transferred aseptically to plates of modified Dox's medium containing 1% pectin as sole carbon source. Three plates were made for each dilution. The plates were incubated for 8 days at 30ºC, and the growing fungal colonies were picked and purified by subculturing on the same medium.

Media for isolation and screening of pectinase producing fungi

Modified Dox´s medium [6]:

Solid media (1% pectin, 0.1%KH_2PO_4, 0.05% $MgSO_4$, 0.3% $NaNO_3$, 0.05% KCl, 2% agar dissolved in tap water) was adjusted to use for screening of pectinase producing potency. The initial pH of the medium was adjusted at 6.

Liquid media was prepared without agar and adjusted to use for screening of pectinase producing potency. The initial pH of the medium was adjusted to 5.5.

Potato-Dextrose agar medium [7]:

250g peeled potato; 20g agar and 20g glucose were dissolved in 1 liter distilled water.

Identification of selected fungal isolate

Morphological characterization of pectinase producing fungi

The fungal isolates were surface plated on modified Dox´s agar and potato-dextrose agar media, incubated at 30°C for 8 days. The mycelia color and exudates pigments were observed and photographed. Microscopic features including conidial heads, sterigmata and conidial ontogeny were also examined **[6].**

Pectinase assay

For screening pectinase enzyme, pectinase activities were measured at 45 °C by Viscometry according to **[8].** For exo-pectinase (exo-p) activity, the release of reducing sugars was detected according to **[9].**

The assay for pectinase enzyme was carried out according to the DNS method's standard methodology **[9].**1 ml crude enzyme was added to 1 ml distilled water, and 1 ml DNS reagent was used in the test. The pectinase assay employed D-galactosamine monohydrate (1 mg/ml) as the standard. At optical density 540 nm, the absorbance readings were taken. The activity of pectinase was measured in enzyme units (U) per ml, with 1 U equaling moles per unit time in minutes. Soluble protein was determined according to the method described by **[10].**

Optimization of cultural conditions for pectinase production

The tested isolate was inoculated in liquid basal medium, some growth factors affecting pectinase production were studied, such as different incubation temperatures (20 to 60ºC) and different pH values (2 to 9); investigation the effect of shaking on pectinases productivity ;different carbon sources introduced separately into basal medium without pectin (carbon source) a control. lactose, xylose, starch, glucose, sucrose, maltose, mannose, fructose and galactose were used at a final concentration of the carbon source 1 w/v. Different organic nitrogen sources (urea, peptone, beef extract, yeast extract and meat extract); inorganic nitrogen sources (ammonium chloride, ammonium nitrate, sodium nitrate and ammonium sulphate) were added to medium at a final concentration equimolecular to that located in 3g/l of $NaNO_3$and different phosphorus sources (KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4, $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$) The strain was grown at different parameters then pectinases productivity, protein content and dry weight were assayed at the end of incubation period.

III. Results and Discussion

A total number of fifty fungal isolates from different sources were checked for their growth on Modified Dox´s medium containing 1% of pectin as a sole carbon for 8 days at 37ºC. Growing fungal isolates were purified and maintained for further studies (Table 1). **[11]** reported pectinase activity of fungi related to different genera.

The enzyme activity of all isolates were determined by viscometer method and showed varying results in agreement with **[12].**Screening pectinolytic activity of 50 fungal isolates by viscometer method at different incubation periods indicated isolate No. 2,isolated from Belbeis soil sample, was the most potent pectinase producing isolate (Table 2). The selected isolate (No.2) was identified according to the universal keys by **[6]** as *Aspergillus niger*.

Results revealed that increasing the incubation temperature enhanced the specific activity of the pectinase enzyme until it reached its maximum value at 30 °C **(Fig 1)**. The present results are in accordance with that obtained by **[13]** where they found the optimal temperature for pectinase production by *Aspergillus spp.* at 30 °C. However, **[14]** found that *Aspergillus niger* gave the highest enzyme yield at 25 °C and **[15]** found that optimal temperature for a heat-tolerant alkaline pectinase from *B. subtilis* ZGL14 was 50°C.

The influence of various initial pH values on pectinase synthesis by the selected isolate was investigated, and it was discovered that the optimal production of pectinase was determined to be at pH 6.0 (**Fig 2)**. **[16]** Reported that optimum pH for the production of pectinase by *Aspergillus oryzae* RR 103 was at pH 6.0. These results contrasted the results obtained by **[17]** who reported that optimum pH values for maximum activity were 11 in case of *Bacillus licheniformis* UNP-1**.**

By studying the effect of static and shaking conditions on activity of produced enzyme. Results showed the highest enzyme specific activity (2.56 U/mg) in static condition **(Fig 3)**.The obtained results are in accordance with that obtained by **[18]** who reported that *A. niger*AUMC4156 was the most promising producer of pectinase under static conditions while *P. oxalicum*AUMC4153 was the highest producer of pectinase under shaken condition.

According to the findings of this investigation, sucrose with a concentration of 0.6 percent (w/v) was the best carbon source giving rise to maximum enzyme activity of pectinase (U/mg) **(Fig 4, 5)**. Our results are in accordance with that obtained by **[19]** who reported that the production of pectinase by a thermophilic *Aspergillus fumigatus* growing the culture in a medium containing sucrose and pectin resulted in the highest levels of enzyme activity. On the other hand, **[20]** found that the maximum pectinase production from *Aspergillus parvisclerotigenus* KX928754 was obtained when allowed to grow in a Czapek-Dox medium containing 1% pectin but devoid of sucrose.

By looking at the impact of various nitrogen sources on the production of pectinase. It was found that yeast extract was the best nitrogen source with concentration 0.8 g/100 mL **(Fig 6, 7)** showing highest enzyme activity (1.454 U/mL). **[21]** Found that yeast extract was the most suitable sources of nitrogen for optimum production of pectinases by *Ralstoniaso lancearum.* On the other hand, **[22]** reported that glutamine; glycine and peptone supported maximum production of pectinase by *Fusarium oxysporum.*

By studying the effect of different phosphorus sources on the production of pectinase synthesis the maximal values were obtained in the presence of Na_2HPO_4 **(Fig 8)** with maximum enzyme activity (1.2 U/mL).That agree with **[23]** for pectinase production by *Bacillus pumilus* dcsr1, **[24]** for pectinase production by *Aspergillus niger* and **[25]** for pectinase production by *Bacillus* species.

Table (1): Isolation of fungi producing pectinase enzyme from different samples on specific medium

Sample No.	Source of collection	Isolate no.	sources of collection	Count of isolates
1	Agricultural soil	1,2	Belbeis	13
		3,4,5,6		
		7,8,9		
		10,11,12		
		13		
2	rotten orange	14,15,16,17	Belbeis	4
3	Agricultural soil	18,19	Mania El Kamh	4
		20,22		
4	Rhizosphere of clover plant	23,24 25	Kafr Ayoub	5
		26 ,27		
5	Rhizosphere of corn plant	28	Belbeis	3
		29		
		30		
6	Rhizosphere of rice plant	31	Kafr Ayoub	3
		32		
		33		
7	sewage water	34	Belbeis	2
8	Agricultural soil	35,36	Kafr Ayoub	8
		37		
		38		
		39		
		40		
		41		
		42		
9	Rotten apple	43	Belbeis	3
		44		
		45		
10	Agricultural soil	46	Zagazig	6
		47		
		48		
		49		
		50		

Table (2): Pectinolytic screening production by *Aspergillus niger* by viscometer method at different incubation periods.

Isolate no.	Incubation period (days)					
	5 Days		10 Days		15Days	
	R1%	R2%	R1%	R2%	R1%	R2%
1	2.74	13.78	2.52	29.17	2.43	1.90
2	49.67	41.51	38	32.03	17.65	15.63
7	35.68	24.9	24.18	19.36	5.57	2.65
8	40.26	26.42	26.48	21.53	15.30	8.79
11	19.43	5.46	18.35	17.33	3.2	5.60
12	20.94	29.83	25.46	37.7	23.51	15.63
13	33.86	13.71	26.69	20.71	12.8	20.70
14	11.14	6.97	10.8	5.53	5.22	4.40
20	46.88	32.63	32.22	17.97	20	16.08
23	44.22	19.8	19.48	7.60	6.58	02.34
24	29.51	4.96	8.09	10.41	14.79	8.2

27	8.91	18.36	19.46	15.81	18.3	29.8
29	9.29	8.91	7.65	5.30	4.46	2.90
30	21.37	17.67	19.30	17.67	9.78	5.80
35	29.88	2.01	6.5	22.5	9.8	2.7
36	8.21	15.75	20.19	35.99	19.02	32.9
38	15.26	13.3	11.37	15.81	3.7	1.90
39	2.74	13.78	2.52	29.17	2.43	2.11
40	20.94	29.83	25.46	37.7	23.51	37.9
41	10.71	18.24	20.62	27.56	25	27.9
42	8.91	18.36	19.46	12.50	18.3	7.80
43	8.21	15.75	20.19	35.99	19.02	32.9
44	10.71	18.24	20.62	27.56	25	2.30
45	18.05	23.95	21.31	27.36	24.02	32.9
46	18.05	23.95	21.31	27.36	24.02	29.8
47	20.23	15.35	17.28	16.44	21.37	17.97
48	24.03	11.8	15.9	13.19	15.26	19.91
49	20.16	6.28	8.7	8.9	8.21	15.34
50	31.18	21.8	19.93	16.76	17.50	10.71

R_1=time of boiling-time of enzyme/time of boiling *100R_2= time of control-time of enzyme/time of control*100

Control=water added instead of enzyme

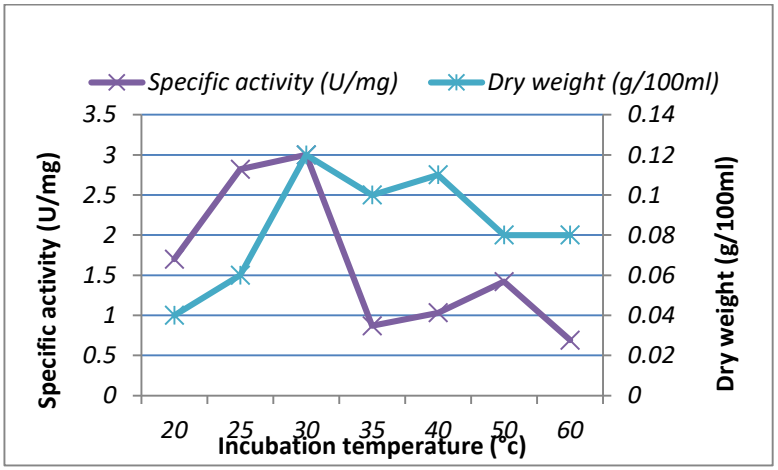

Fig (1): Pectinase production at different incubation temperatures by *Aspergillus niger*

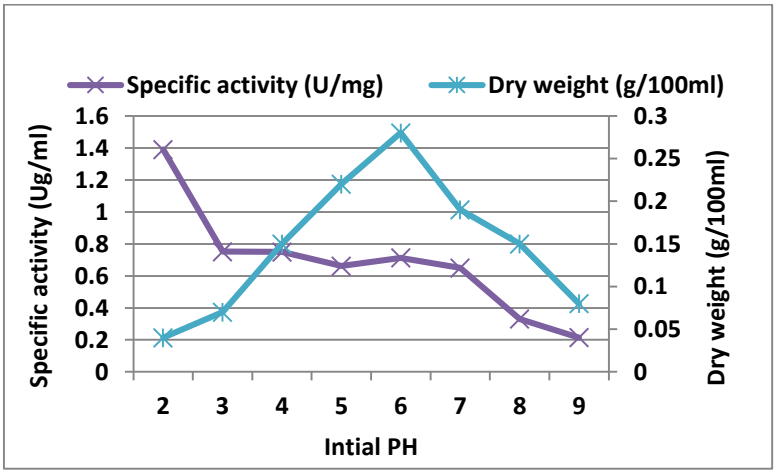

Fig (2): Pectinase production at different initial pH values by *Aspergillus niger*

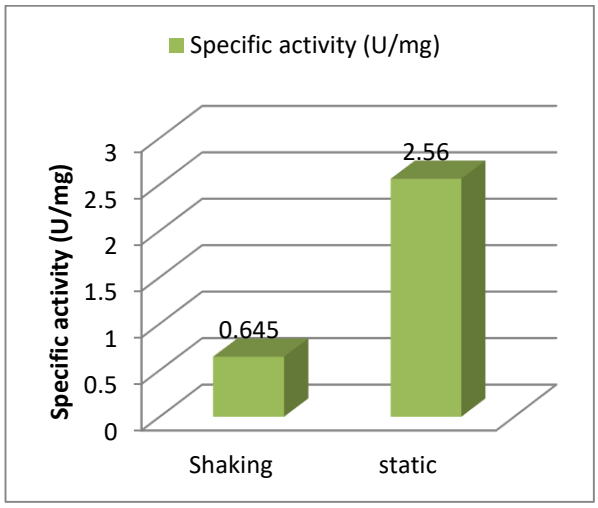

Fig (3): Pectinase production at static and shaking conditions by *Aspergillus niger*

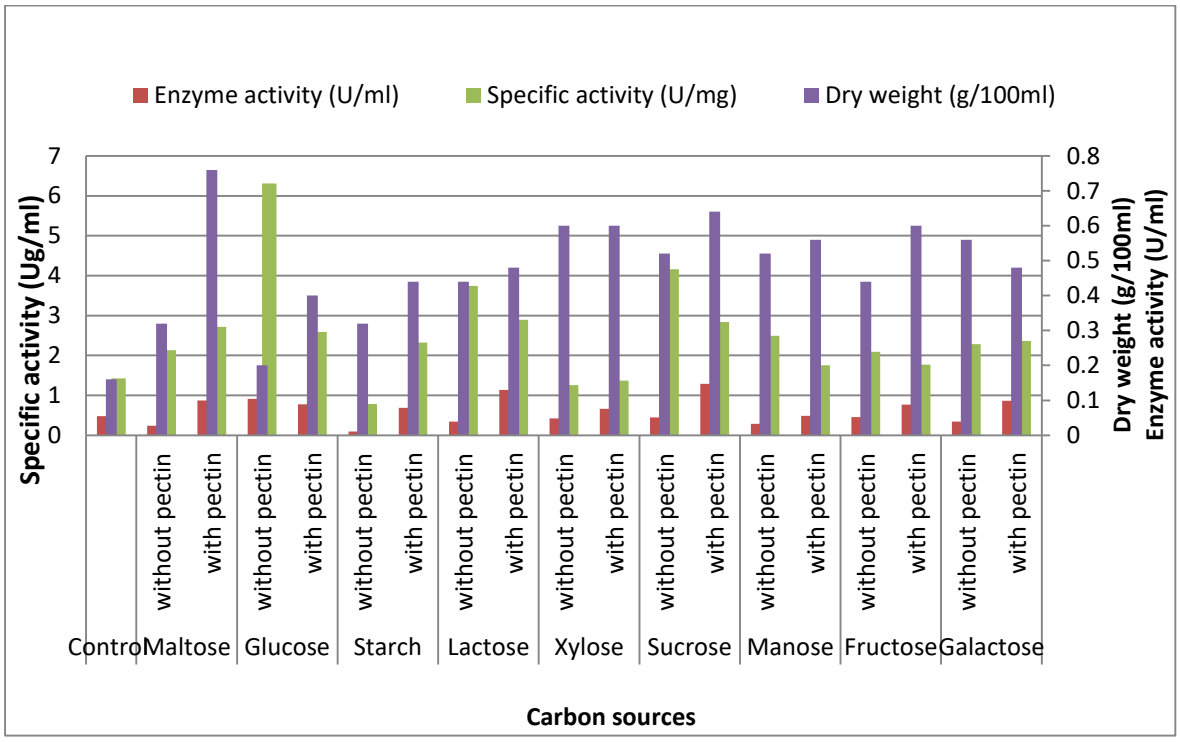

Fig (4): Pectinase production at different carbon sources by *Aspergillus niger*

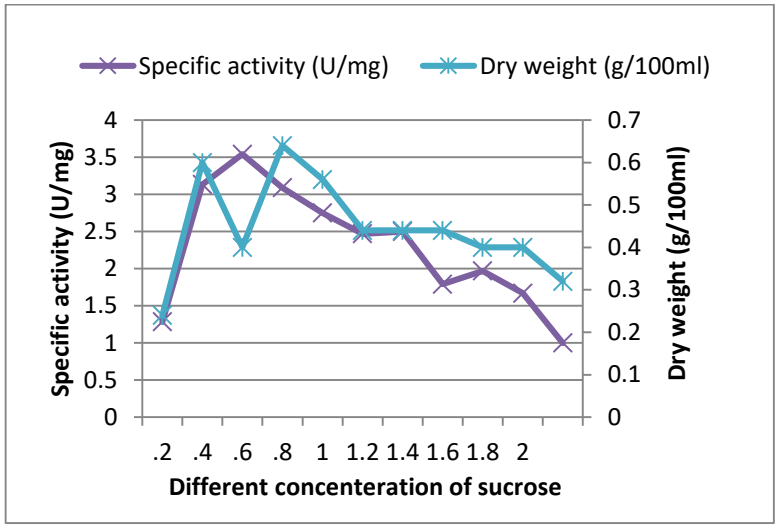

Fig (5): Pectinase production at different concentration of sucrose by *Aspergillus niger*

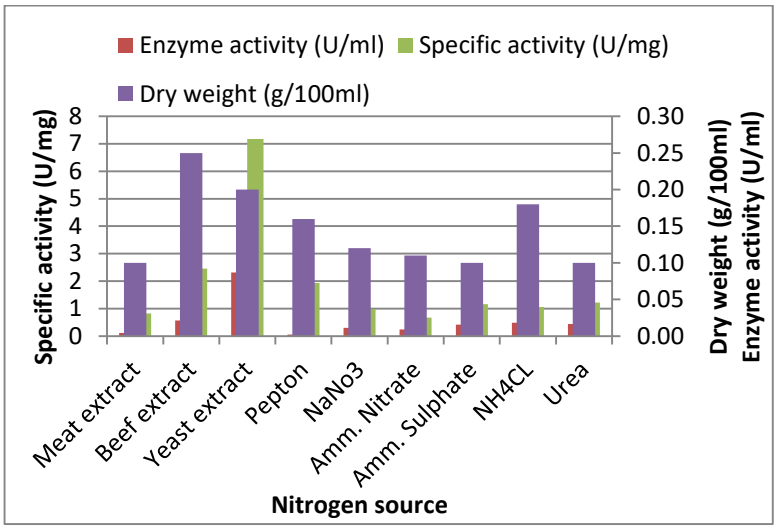

Fig (6): Pectinase production at different nitrogen sources by *Aspergillus niger*

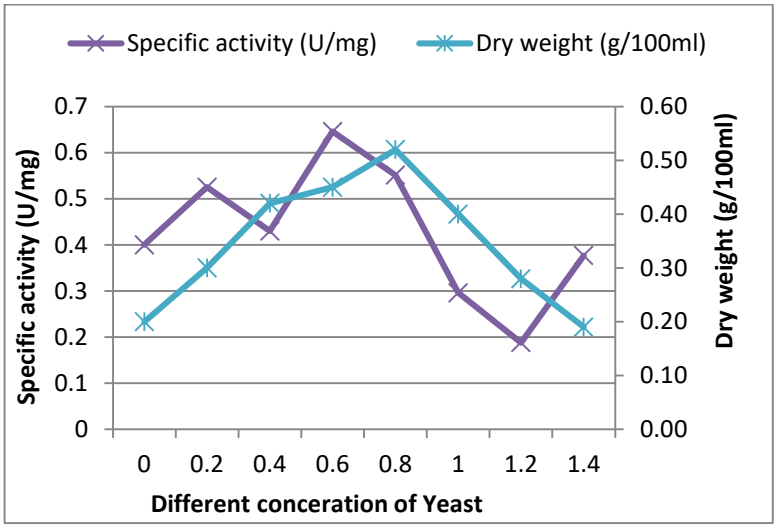

Fig (7): Pectinase production at different concentration of yeast by *Aspergillus niger*.

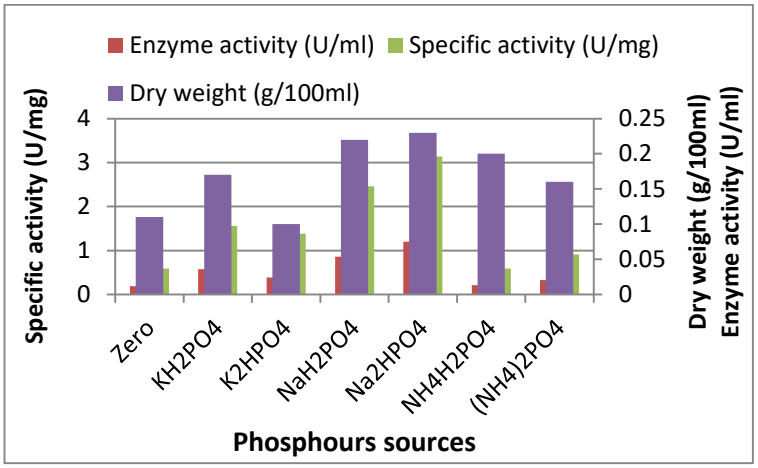

Fig (8): Pectinase production at different phosphorus sources by *Aspergillus niger*.

IV.CONCLUSION

The highest production of pectinase enzyme by local isolate *Aspergillus niger* was obtained after 4 days of incubation at 30ºC and medium was adjusted to pH 6, supplemented with addition of yeast (0.8 g/100 mL), sucrose (0.6 g/100 mL) and Na_2HPO_4.

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