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Improvement of Tannase Production from Bacillus Bacteria by Submerged Fermentation of Spent Tea

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تحسين ظروف إنتاج الإنزيم المحلل للتانينات من البكتريا العصوية عن طريق التخمير المغمور لثفل الشاي

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ABSTRACT

Tannase is an enzyme that causes hydrolysis of a group of tannins (gallo-tannins) to gallic acid and glucose. This enzyme is of importance due to its numerous applications in many fields, such as in the food industry by enhancing tea and coffee flavor and improving the quality of fruit juices rich in tannins. Tannase is also of importance in improving the quality of animal feed. Gallic acid is applied in the drug industry, and the production of antioxidants is used in the oil industry. This study was carried out to improve production of tannase from genus *Bacillus* using spent tea as a substrate by submerged fermentation and applying the statistical design Response Surface Methodology (RSM). Five parameters were considered, and each parameter was studied at five levels. They were temperature range (25 to 45°C), pH (3–8), incubation period (24–120 hours), spent tea (0.5–2.5%) and rotation speed (100–300rpm). Results obtained revealed that optimum conditions for maximum production of tannase were: temperature at 35°C, pH at 5, incubation time of 48 hours, spent tea concentration 1.5%, and rotation speed of 150 rpm. We recommend that further studies be carried out to clarify and determine the properties of crude and purified enzymes.

الملخص

يعمل الإنزيم المحلل للتانينات Tannase على تحليل التانينات من مجموعة gallo-tannins إلى حمض الغاليك وغلوكوز، وتعود أهمية هذا الإنزيم للتطبيقات الواسعة في عدة مجالات منها الصناعات الغذائية كتحسين نكهة الشاي والقهوة ، تحسين جودة عصائر الفاكهة الغنية بالتانينات إضافة إلى أهميته في تحسين نوعية أعلاف الحيوانات، كما يستخدم حمض الغاليك الصناعات الدوائية وإنتاج مضادات الأكسدة المستخدمة في صناعة الزيوت، لذا تمت دراسة إنتاج إنزيم التاناز من جنس بكتريا Bacillus باستخدام ثفل الشاي كركيزة باستخدام التصميم الإحصائي Response Surface (RSM) Methodology، لتحسين ظروف إنتاج إنزيم التاناز بطريقة التخمير المغمور. درست خمسة متغيرات بخمسة مستويات لكل منها كالتالي لدرجات الحرارة (25-45°م) ودرجة الحموضة pH (3-8) ومدة التحضين(24-120 ساعة) وتركيز ثفل الشاي (0.5-2.5%) كانت الشروط المثالية لتحسين إنتاج الإنزيم عند درجة الحرارة 35°م، و5 pH، ومدة تحضين 48 ساعة ، وثفل الشاي 1.5% ، و سرعة دوران 150د/د. نوصي بالقيام بدراسات لاحقة لتنقية الإنزيم وتحديد خصائص كل من الإنزيم الخام والمنقى.

1. Introduction

Tannases (tannin acyl hydrolase EC 3.1.1.20) are hydrolytic enzymes that catalyze the hydrolysis of ester bonds in hydrolysable tannins, such as tannic acid, and the release of glucose and gallic acid (3,4,5-trihydroxy benzoic acid) (Van de Lagemaat and Pyle, 2005).

Tannase is used in the manufacturing of instant tea and coffee flavoring (Aguilar et al., 2007), and it is used in producing gallic acid, a substrate for propyl gallate production and trimethoprim synthesis (Bajpai and Patil, 2008). Tannase is also applied in beer and wine industries to remove chill haze formation (Vaquero et al., 2004; Aithal and Belur, 2013), and it is also used to reduce the anti-nutritional effects of poultry and animal feed along with food de-tanification (Xu., 2009) and industrial tannery effluent treatment (Orlita, 2004).

Many studies indicated plants and animals as sources for production of tannase in addition to microorganisms like bacteria, fungi, and yeasts (Chavez -Gonzalez et al., 2011; Aguilar-Z´arate et al., 2014). Species of *Aspergillus*, *Penicillium*, *Rhizopus*, *Paecilomyces*, *Lactobacillus* and *Klebsiella pneumoniae* and *Citrobacter freundii* (Kumar et al., 1999) have been reported to produce the enzyme through fermentation (Mondal et al., 2001). Some species of *Bacillus* have also been found to produce considerable amounts of tannase in broth culture, such as *B. cereus* and *B. licheniformis*, and it was reported to be an inducible enzyme (Mondal et al., 2001; Maity et al., 2009).

Microbial tannase enzymes are the most preferred source of commercial enzymes because they are more stable than those extracted from other sources (Jana et al., 2014), in addition to their economic value by utilizing renewable resources and the ability to improve enzyme production by controlling production conditions (Vikas et al., 2013; Belur and Mugeraya, 2011). Microbial production of tannase is carried out through solid, liquid surface, and submerged fermentation, as the enzyme is extracellular and is secreted directly into the medium (Lekha and Lonsane, 1997). Production of tannase from a bacterial source usually requires submerged fermentation for enhancing yield due to the ease of its application and the simplicity in enzyme extraction and separation from the biomass in addition to the reduced period of time needed for the fermentation process (Prasad et al., 2012).

Optimization of cultural conditions during fermentation results in a considerable increase in the production of tannase by microorganisms (Belmares et al., 2004). A factor affecting tannase production positively is the use of various tannin-rich substrates like sugarcane baggase and wheat bran, plus Jamun leaves (*Syzygium cumini*) and Creosote bush leaves have also been applied (Kumar et al., 2007; Trevino-Cueto et al., 2007). Methods for optimization of medium and fermentation conditions are time consuming and expensive since each experiment involves varying one parameter and keeping the others constant. Other factors that need to be addressed are the interactions among various physicochemical parameters. To overcome these difficulties, a statistical method was applied known as Response Surface Methodology (RSM). This method helped to evaluate and understand the interactions between different physiological and nutritional parameters (Saxena and Saxena, 2004; Naidu et al., 2008; Belur and Mugeraya, 2011). RSM has an advantage over traditional methods by optimizing the parameters that influence maximum yield and higher enzyme activity at a lower

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cost (Belur and Mugeraya, 2011).

A major factor that limits application of tannase enzyme in industry is its high cost and the small number of studies carried out concerning the physical and chemical properties of this enzyme. Production of tannase in laboratories was accomplished by methods of small-scale fermentation using flasks and shaker incubators from which optimal conditions for production were determined. Production methods were developed to obtain a higher yield of enzyme at the lowest cost (Sharma et al., 2014). Pure tannic acid was previously used in tannase production as a sole carbon source, but it was of high cost and did not suit enzyme production at an economical level. The best solution was the use of crude tannins in tannase production (Selwal et al., 2011). Their main sources were agricultural wastes, forest wastes, and factory wastes (olive pomace, grape pomace, pomegranate peels, coffee pulp, tea residue, etc.), which are considered to be the best sources of tannin-rich substrates (Sharma et al., 2014; Bhoite and Murthy, 2015). Nandini et al. (2014) produced tannase using several substrates among which was spent tea by *Bacillus* sp. Selvaraj and Murty (2017) produced tannase by *Bacillus gottheilii* using tea leaves. In a recent study, Unban et al. (2020) produced tannase from a tannase producing strain of *Bacillus* using fermented tea leaves.

The aim of this study was to optimize conditions for tannase production from *Bacillus* sp using spent tea as substrate by applying an RSM statistical program.

2. Materials and Methods

2.1. Microorganism Culture and Maintenance

Bacillus sp was used in the current study. This isolate was isolated from soil samples of an olive field in Latakia, Syria in 2017. Bacteria was classified to the genus level according to Bergey's Manual (Vos et al., 2009).

The bacterial isolate used in this study was selected from among 30 isolates that produced tannase. The selected isolate was grown and maintained on nutrient agar (HiMedia, India) plates containing 0.5% tannic acid (Sigma Aldrich, China) filter sterilized as a substrate and sole source of carbon, plates were incubated at 30°C for 48 hours (Brahmbhatt and Modi, 2015).

2.2. Tannase Production

Tannase production was carried out in 250 ml Erlenmeyer flasks with 50 ml of fermentation medium consisting of (g/l): 3 $NH4NO_3$, 0.5 KH_2PO_4, 0.5 K_2HPO_4, 1 $CaCl_2$, 0.5 $MgSO_4$ (Aftab et al., 2016). And spent tea as a substrate and sole source of carbon was added according to the approved statistical design in the following concentrations: 0.5, 1, 1.5, 2, 2.5%. The medium was then autoclaved at 121°C for 15 minutes, then it was cooled and inoculated with 2 ml of overnight inoculum that was prepared in nutrient broth with 0.5% tannic acid and incubated at 30°C (Aftab et al., 2016). Individual fermentation experiments were carried out under different fermentation conditions of incubation temperature, incubation time, pH, and aeration speed. Parameters were then chosen according to Table (1).

2.3. Determination of Tannase Activity

The fermentation product was centrifuged at 10,000 rpm for 10 minutes at 15°C, then it was filtered, and an estimation of tannase activity was assayed (Brahmbhatt and Modi, 2015).

Tannase was assayed by the method based on chromogen formation between gallic acid and rhodanine (2-thio-4-ketothiazolidine). An enzyme sample (0.25 ml) of culture filtrate and a substrate solution of methyl gallate 0.05M (0.25 ml) were pre-incubated at 30°C for 5 minutes, then 300 µL of 0.667% methanolic rhodanine (Sigma Aldrich, Germany) was added to the mixture and incubated for 5 minutes at 30°C. This was followed by the addition of 200 µL of KOH (0.5M). The mixture was then diluted to 5 ml by adding distilled water. After an incubation of another 10 minutes at room temperature, absorbance at 520 nm was measured by spectrophotometer. One unit of tannase activity is defined as the amount of enzyme required to release 1 µmol of gallic acid per minute under standard assay conditions (Sharma et al., 2000; Fang et al., 2019).

2.4. Optimization of Fermentation Conditions Using RSM

Determination of optimal conditions for tannase production using spent tea as substrate was investigated by studying the effect of five variable factors (incubation temperature, pH, incubation period, aeration speed, and substrate concentration) on tannase activity, where production is determined in terms of enzyme activity.

The statistical design for the experiments performed was Response Surface Methodology (RSM) (Raghuwanshi et al., 2011; Tripathi and Lakshmi., 2018) using a Minitab optimization method in order to study the main factor effect and the effect of interaction among factors. Each variable was studied at five levels (+2, +1, 0, -1, -2) as follows in Table (1). The design included 32 experimental plots as shown in Table (2).

Table (1): Levels of the five independent variables (factors) used in RSM

Variables	Unit	-2	-1	0	+1	+2
incubation temperature	℃	25	30	35	40	45
pH		4	5	6	7	8
incubation period	Hours	24	48	72	96	120
aeration speed	rpm	100	150	200	250	300
substrate concentration	%	0.5	1	1.5	2	2.5

The relationship between the enzyme activity, (Y):Response, and the five studied variables, (X):Independent variables, was expressed by a quadratic equation as follows:

$$Y=a+bX_1+cX_2+dX_3+eX_{4+}fX_5+gX_1^2+hX_2^2+iX_3^2+jX_4^2+kX_5^2+lX_1X_2+mX_1X_3+nX_1X_{4+}oX_1X_5+pX_2X_3+qX_2X_4+rX_2X_5+sX_3X_4+tX_3X_5+uX_4X_5$$

Where *a* is constant, *b,c,d,e,f* are linear coefficients, *g,h,i,j,k* are square coefficients, and *l,m,n,o,p,q,r,s,t,u* are interaction coefficients.

3. Results and Discussion

According to the statistical design, thirty-two experiments were carried out in duplicate, and tannase activity was determined as shown in Table (2), which represents the results of tannase activity produced from *Bacillus* sp using spent tea as a substrate.

Table (2): Enzymatic activity using parameters in accordance to the approved statistical design

Blocks	Temperature ℃	pH	Incubation time hours	Aeration speed rpm	Substrate %	Tannase activity U/ml	
						1	2
1	30	5	48	150	2	0.315	0.315
2	40	5	48	150	1	0.120	0.123
3	30	7	48	150	1	0.240	0.222
4	40	7	48	150	2	0.039	0.054
5	30	5	96	150	1	0.273	0.285
6	40	5	96	150	2	0.120	0.135
7	30	7	96	150	2	0.216	0.213
8	40	7	96	150	1	0.045	0.051
9	30	5	48	250	1	0.270	0.276
10	40	5	48	250	2	0.138	0.126
11	30	7	48	250	2	0.234	0.237
12	40	7	48	250	1	0.099	0.102
13	30	5	96	250	2	0.252	0.258
14	40	5	96	250	1	0.126	0.129
15	30	7	96	250	1	0.213	0.249
16	40	7	96	250	2	0.033	0.030
17	25	6	72	200	1.5	0.114	0.108
18	45	6	72	200	1.5	0.063	0.072
19	35	4	72	200	1.5	0.336	0.336
20	35	8	72	200	1.5	0.222	0.222
21	35	6	24	200	1.5	0.198	0.201
22	35	6	120	200	1.5	0.063	0.066
23	35	6	72	100	1.5	0.318	0.321
24	35	6	72	300	1.5	0.306	0.312
25	35	6	72	200	0.5	0.300	0.300
26	35	6	72	200	2.5	0.255	0.249
27	35	6	72	200	1.5	0.327	0.321
28	35	6	72	200	1.5	0.321	0.324
29	35	6	72	200	1.5	0.321	0.315
30	35	6	72	200	1.5	0.324	0.327
31	35	6	72	200	1.5	0.318	0.321
32	35	6	72	200	1.5	0.324	0.27

Table (3) shows the effect of the studied factors (individual factors, the square of factors, and the interaction of factors) on the activity of tannase.

Statistical analysis of the effect of the five studied variables (temperature, pH, incubation time, aeration speed, and substrate concentration) separately on the activity of tannase is shown in Table (3). It was noted that the P values for temperature, pH, substrate concentration, and incubation time were lower than 0.05 ($P<0.05$) indicating there was a significant linear effect for each of these variables on the production of the tannase enzyme; conversely, the P value for the aeration speed was greater than 0.05 ($P>0.05$), and this indicated that this factor had no significant effect on tannase production.

Studying the quadratic relations of the variable factors on tannase production showed the following results as observed in Table (3) where the P value for temperature, pH, incubation time, and substrate concentration were lower than 0.05 ($P<0.05$). These results indicate the presence of significant effects of these factors on the enzymatic activity; hence, the relationship between these variables and enzyme activity could be plotted as Parabola.

The interaction effect among variables, as shown in Table (3) and Figure (1), showed that the interaction relationships between temperature with both pH and incubation time were significant in influencing enzyme activity because the P values were lower than 0.05 ($P<0.05$). With the rest of the relationships, P was greater than 0.05 ($P>0.05$); therefore, its effect was not significant on enzyme activity and, hence, production.

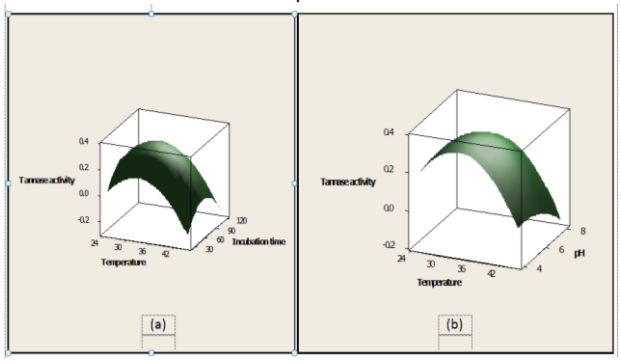
The determination coefficient R^2 was 89.50% indicating that the regression equation for the variable factors affected 89.50% of change in enzyme activity.

Table (3): Statistical analysis of the variation of studied variables that affected tannase activity

Term	Coef	SE Coef	T	P
Constant	0.180909	0.046485	3.892	0.000
Temperature	-0.071042	0.022064	-3.22	0.002
pH	-0.05375	0.022064	-2.436	0.019
Incubation time	-0.030208	0.022064	-1.369	**0.008**
Aeration speed	-0.021458	0.022064	-0.973	0.336
Substrate	-0.175303	0.063951	-2.741	0.009
Temperature*Temperature	-0.061136	0.005241	-11.665	0.000
pH*PH	-0.013011	0.005241	-2.483	0.017
Incubation time*Incubation time	-0.049886	0.00524	-9.518	0.000
Aeration speed*Aeration speed	-0.004261	0.00524	-0.813	0.421
Substrate*Substrate	-0.054545	0.020964	-2.602	0.013
Temperature*pH	-0.004375	0.007096	-0.617	**0.041**
Temperature*Incubation time	0.00125	0.007096	0.176	**0.011**
Temperature*Aeration speed	0.00625	0.007096	0.881	0.383
Temperature*Substrate	-0.00875	0.014193	-0.617	0.541
pH*Incubation time	-0.001875	0.007096	-0.264	0.793
pH*Aeration speed	0.006875	0.007096	0.969	0.338
pH*Substrate	-0.015	0.014193	-1.057	0.297
Incubation time*Aeration speed	-0.00375	0.0071	-0.528	0.600
Incubation time*Substrate	-0.00875	0.014193	-0.617	0.541
Aeration speed*Substrate	-0.01375	0.014193	-0.969	0.338

Coef: coefficients, SE Coef: standard error of the coefficient, P: probability
R2 = 89.50%

Figure (1): Surface plot of tannase activity vs. (a) incubation time and temperature (b) pH and temperature



According to Table (3), the equation could be written as follows:

$$Y=0.180909-0.071042X_1-0.05375X_2-0.030208X_3-0.021458X_4-0.175303X_5-0.004375X_1X_{2+}0.00125X_1X_3+0.00625X_1X_{4-}0.00875X_1X_50.001875X_2X_3$$

$$+0.006875X_2X_4-0.015X_2X_5-0.00375X_3X_4-0.00875X_3X_5-0.01375X_4X_5-0.061136X_1^2 \quad -0.013011X_2^2 \quad -0.049886X_3^2 \quad -0.004261X_4^2 -0.054545X_5^2$$

X_1: Temperature, X_2: pH, X_3: Incubation time, X_4: Aeration speed, X_5: Substrate concentration.

Table (4): Analysis of variance for tannase activity

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	20	0.574	0.574	0.029	17.810	0.000
Linear	5	0.224	0.043	0.009	5.330	0.001
Square	5	0.342	0.342	0.068	42.420	0.000
Interaction	10	0.009	0.009	0.001	0.530	0.045
Residual Error	42	0.068	0.068	0.002		
Lack-of-Fit	32	0.065	0.065	0.002	8.440	0.121
Pure Error	10	0.002	0.002	0.000		
Total	63	0.642				

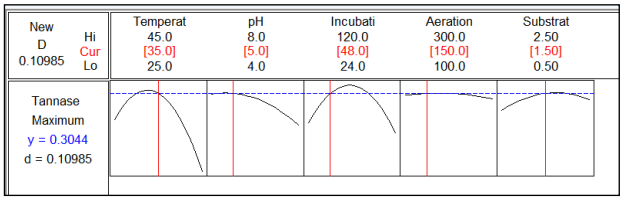
DF: degrees of freedom, Seq SS: sequential sums of squares; Adj SS: adjusted sums of squares, Adj MS: adjusted mean squares; F: frequency, P: probability

Table (4) shows the statistical analysis of the variance of the studied variables that affected the production of the enzyme. The presence of at least one linear variable within the five single variables indicates significant effect because ($P<0.05$). There was also at least one square variable of the five square variables showing significant effect ($P<0.05$), and among the ten correlations, there was at least one significant single interaction where ($P<0.05$).

Figure (2) shows the optimal conditions for tannase production from *Bacillus* sp using spent tea as a substrate, which were as follows according to the statistical results: temperature at 35°C, pH at 5, incubation time at 48 hours, aeration at 150 rpm, and substrate concentration 1.5%.

When these conditions were applied, the enzymatic activity was 0.299 units/ml, which was close to the theoretical enzyme activity (y=0.304) value estimated by RSM.

Figure (2): Optimal conditions for the production of tannase enzyme from *Bacillus* sp using spent tea as a substrate by the RSM statistical test.



Results shown in Figure (2) indicate a continuous increase in enzyme activity with temperature until it reaches 35°C, which proved to be the optimum temperature for enzyme production when using spent tea as substrate. An increase in incubation temperature above 35°C revealed a gradual decrease in enzyme activity (production) due to the negative effect of higher temperature on bacterial growth, which affects enzyme production. The obtained results are in agreement with those obtained by Das Mohapatra et al. (2006), in that optimal temperature for tannase production from *Bacillus licheniformis* KBR6 using barks of different forest plants as substrate was 35°C. This also agrees with results obtained by Jana et al. (2013), who mentioned that optimal temperature for tannase production from *B. subtilis* PAB2 was 35°C using tannic acid as substrate . The optimum pH for tannase production from *Bacillus* sp under optimal experimental conditions using spent tea as substrate was pH 5. These results coincide with results obtained by Mondal and Pati (2000) when producing tannase from *Bacillus licheniformis* KBR6. Determining incubation time is an important and critical factor that affects reaching optimal enzyme production. Its importance comes from the effect of time on bacterial growth and, hence, enzyme production. In Figure (2), an increase in enzyme activity is observed along with the increase in incubation time until a maximum value of

enzyme activity is reached after 48 hours of incubation, after which, a decline in enzyme activity is noticed as incubation hours increased.

Enzyme activity reaches its lowest level after 120 hours of incubation, where growth declines due to depletion of nutrients and formation of inhibitors by bacteria as well as accumulation of glucose and gallic acid in the medium. These results agree with those obtained by Selvaraj and Murty (2017) when producing tannase from *Bacillus gottheilii M2S2* using *Triphala* plants as substrate. When producing tannase by *Bacillus sphaericus* with tannic acid as substrate, optimal incubation time was 48 hours (Raghuwanshi et al., 2011). In another study carried out by Tripathi et al. (2016) on the production of tannase from tannic acid as substrate by *Bacillus megaterium*, optimum incubation time was 72 hours. The variation in results concerning optimum incubation time may be due to the difference in the chemical composition of the different substrates applied, especially those related to tannin ratio and other differences among bacterial species and temperatures and pHs applied. All these factors affect optimal incubation time in enzyme production. Different rotation speeds were applied using a shaker incubator when incubating fermentation flasks to determine the optimal speed for aeration in tannase production. It was observed that highest enzyme activity was obtained when applying a speed of 150 rpm. A number of results from several studies were comparable with our result even though different bacteria were applied, such as *S.ficaria* and *Enterobacter cloacae*, when optimizing conditions for tannase production (Belur et al., 2010; Beniwal et al., 2010). Many studies indicated that using an aeration speed lower than 100 rpm does not provide convenient mixing of fermentation medium constituents during the fermentation process.

Results shown in Figure (2) also indicate that using spent tea at a concentration of 1.5% (w/v) in fermentation medium was the best to reach optimum conditions in tannase production. Determination of optimal concentration of tannic acid in tannase production from *Bacillus* sp varied among studies conducted for this purpose, where Banerjee et al. (2001) mentioned that tannic acid had to be in a concentration range between 0.25–1.25% in medium because it has a role in inducing enzyme production. In another study by Mondal et al. (2000), it was found that optimal concentration of commercial tannic acid when producing tannase by *Bacillus licheniformis* was 1.5% (w/v) in fermentation medium. Raghuwanshi et al. (2011) also mentioned that the optimal concentration of tannic acid was 1.5% (w/v) in fermentation medium when producing tannase from *Bacillus sphaericus*. In another study conducted by Aissam et al. (2005), results showed that an increase in tannic acid concentration above 3.5% (w/v) in fermentation medium resulted in the formation of irreversible bonds between cell surface proteins, which affected food metabolism and bacteria growth, and, hence, reduced tannase production. Seth and Chand (2000) also mentioned that utilization of a high concentration of gallic acid caused a decrease in tannase production due to accumulation of gallic acid on the bacterial cell surface.

4. Conclusion

We were able to optimize conditions for tannase production from *Bacillus* sp using spent tea as substrate to describe the effect of different parameters as designed by the statistical analysis program (RSM). From results obtained, optimum conditions for production of tannase were found to be at an incubation temperature of 35°C, pH 5, incubation time at 48 hours, aeration speed at 150 rpm, and spent tea concentration of 1.5% in fermentation medium.

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