Original Research Article

Factorial design based bench-scale production of collagenase by *Pseudomonas* sp. found in protein waste of Himalayan region

Abstract

Collagenase is commercially important and reported from *Pseudomonas* sp. found in protein waste of Himalaya by our group. Therefore, current study was focused on factorial design based bench-scale production of collagenase by *Pseudomonas* sp. Chemical and fermentation conditions including medium contents (carbon, nitrogen, and growth supplements) were optimized and found that sucrose, tryptone and gelatin substrate stimulates the production of collagenase. Factorial (2^6) design was used for maximum collagenase production and maximum 1.43 U/mL increase was obtained with 57^{th} combination of factorial design. The bench-scale production of collagenase was achieved in a 6 L working volume laboratory fermenter. The different sets of agitation speed, aeration rate were optimized to enhance the economical production of collagenase by *Pseudomonas* sp. The bench-scale fermenter produced 2.3-fold enhanced collagenase activity at reduced cultivation time (14^{th} h) compared to the shake flask (24^{th} h).Further factorial design is worthful for the production of collagenase.

Keywords: Collagenase; Protein waste; *Pseudomonas;* Factorial design; Laboratory fermenter

1.0 Introduction

Collagen is the major fibrous component in animal and human extracellular connective tissues. It is mostly found in skin, bone, tendon, teeth and blood vessels. The degradation of collagen produces peptides which play major role in several industrial and medical applications [1]. Collagenases are capable of degrading the polypeptide backbone into peptides. The collagenases are majorly two types (Serine and metallocollagenases) which play important role in several physiological functions. Serine collagenases are probably involved in the production of hormones and pharmacologically-active peptides, as well as in various cellular functions. These functions include protein digestion, blood-clotting, fibrinolysis, complement activation and fertilization [2-3]. The molecular weight of these enzymes was reported in the range of 24-36 kDa [4]. On the other hand, metallocollagenases

are zinc-containing enzymes which require calcium for the stability [5]. These metallocollagenases (extracellular enzymes) are involved in remodelling of the extracellular matrix, and their molecular weights vary from 30 to 150 kDa [6-10]. However, the majority of connective tissue destruction was reported by matrix metalloproteinases [11-15]. Earlier our group has reported the screening, isolation, characterization, and purification of collagenase from *Pseudomonas* sp. found in protein waste of Himalayan region [16-17]. Further, the intact cuticles of fish nematode and plant root-knot nematode *Meloidogyne javanica*were digested by collagenase produced by Pseudomonas sp. The degradation of cuticular proteins can be used for controlling pre- and post- parasitic forms of nematodes [18]. It was observed that yield of the extracellular enzymes were significantly influenced by physicochemical conditions. Therefore, optimization of parameters for the large-scale production of collagenase by *Pseudomonas* sp. is required. Thus, an attempt has been made to optimize physiochemical parameters in order to produce maximum amount of collagenase.

2.0 Materials and Methods

Collagenase producing microorganism (*Pseudomonas* sp) was used for the bench-scale production of extracellular collagenase, which was earlier screened, isolated, purified and characterized by our group from the soil/sewage samples collected from the local fish market and slaughterhouse area of Bilaspur and Shimla, Himachal Pradesh, India. The 14 L fermenter (Scigenics India Pvt. Ltd.) with a working volume of 6 L was also used for the study. The fermenter was well equipped with pH, temperature, agitation, aeration, and dissolved oxygen sensors and controls. The effect of aeration rate and agitation rate on cell growth, collagenase production and other parameters such as pH, dissolved oxygen (DO; % saturation) were determined during the fermentation of *Pseudomonas* sp. The various physicochemical parameters were optimized for the production of maximum amount of collagenase by *Pseudomonas* sp.

2.1 Optimization of the individual medium component for the production of extracellular collagenase by Pseudomonas sp.

In order to check the role of individual component of the selected M-5 medium [(pH 6.5), containing (%, w/v; sucrose 1.0, peptone 1.0, yeast extract 0.2, Na_2HPO_4 0.2, Na_2CO_3 0.25, and $MgSO_4.7H_2O$ 0.04)]on the growth and production of collagenase by *Pseudomonas* sp., each medium components were added separately to the production media containing gelatin as inducer.

2.1.1 Carbon sources

Various carbon sources (dextrose, fructose, maltose, sucrose, lactose, galactose, mannitol, glycerol, starch, and xylose) at a concentration of 1% (w/v) in the production medium were used to check their effect on the growth and production of collagenase by *Pseudomonas* sp.

2.1.2 Nitrogen sources

Organic nitrogen sources (peptone, tryptone, urea, soybean meal extract, soypeptone, and casein) were used for the growth and production of collagenase by *Pseudomonas* sp. at a concentration of 1% (w/v).

2.1.3 Growth supplements

For the maximum growth and production of collagenase by *Pseudomonas* sp., various growth supplements (yeast extract, malt extract, meat extract and beef extract) were used individually at a concentration of 0.2% (w/v) in the production medium.

2.1.4 Additional growth supplements

For the assessment of the combinatorial effect of growth supplements at a concentration of 0.25 (%, w/v) on collagenase production by *Pseudomonas* sp., the growth supplements (malt extract, meat, and beef extract) were added in combination with yeast extract (0.25%, w/v).

2.2 Factorial based technological combinations (2^6) of optimized physicochemical parameters

Technological combinations were designed to obtain the best combination of physical and chemical factors for the maximum production of collagenase by *Pseudomonas* sp. The physical factors considered were medium pH, incubation temperature and chemical factors include the concentration of sucrose, tryptone, yeast extract and meat extract. In these sets of experiments, instead of one parameter being varied, different combinations of optimum and next nearest level of optimized parameters were used. In each case, growth, final pH and collagenase production by *Pseudomonas* sp. were monitored. Total 64 combinations (2^6) were obtained by above parameters.

2.3 Bench scale production of Pseudomonas sp. in a laboratory scale fermenter

The Bench scale production of *Pseudomonas* sp. was done at a scale of 6 L working capacity of 14 L laboratory-scale fermenter**.** For the development of a laboratory inoculum, seed medium was inoculated with *Pseudomonas* sp. and incubated at 37^{o}C for 21h on a rotary shaker (150 rpm). The production medium (pH 7.0) contained (%, w/v; sucrose 1.0, tryptone 1.0, yeast extract 0.25, meat extract 0.2 and gelatin 0.3) was loaded to the fermenter with additionally contained 0.01% (v/v) silicone oil (Hi-media) as antifoam agent. The growth of *Pseudomonas* sp. and activity of collagenase was measured under different conditions of

agitation and aeration. The effect of these variables on pH, dissolved oxygen (DO, % saturation), cell mass and collagenase activity was observed.

2.4 Effect of agitation on the growth and production of collagenase by Pseudomonas sp.

The growth of *Pseudomonas* sp., collagenase activity, DO (% saturation) and pH of the fermentation broth was investigated using the varying agitation rate (150, 300 and 450 rpm). The fermentation was carried out at 37^{o}C with constant aeration rate at 0.25 vvm. Samples at regular interval of 2 h were withdrawn and analysed for the growth and production of collagenase by *Pseudomonas* sp. The pH and DO (% saturation) of the fermentation broth during the entire course of fermentation were monitored with the help of DO and pH probe.

2.5 Effect of aeration rate on growth and production of collagenase by Pseudomonas sp.

The effect of aeration rate on the growth and production of collagenase by *Pseudomonas* sp. was also studied under varying aeration rates (0.25, 0.50 and 0.75 vvm) at 300 rpm agitation. The change in pH and DO (% saturation) profile of the fermentation broth was monitored.

2.6 Course of cultivation of Pseudomonas sp. in a bench scale fermenter

The production medium (pH 7.0) containing (%, w/v) sucrose 1.0; tryptone 1.0; meat extract 0.25; yeast extract 0.2 and gelatin 0.3 was inoculated with old seed culture (21 hour;4 % v/v) and incubated at 37^{o}C at the agitation speed of 300 rpm and aeration rate of 0.50 vvm. The course of cultivation of *Pseudomonas* sp. was observed up to 24 h.

3.0 Results and Discussion

3.1 Optimization of various factors for the production of extracellular collagenase by Pseudomonas sp.

3.1.1 Carbon sources

Among the various carbon sources, sucrose was found most important for the growth and production of collagenase (0.557 U/mL) by *Pseudomonas* sp. as compared to control (0.218 U/mL). Different concentrations of sucrose (0.25-2.50%, w/v) were used to select the most appropriate concentration for the maximum growth and production of collagenase from *Pseudomonas* sp. (Fig.1). The addition of sucrose at 1.25% (w/v) concentration was found most suitable for growth and collagenase production (0.567 U/mL) by *Pseudomonas* sp. Jain and Jain, (2010) reported that addition of soluble starch in the production medium supported the growth and production of collagenase by *S. exfoliatus*. However, various carbon sources reported to repress the synthesis of collagenase by *A. iophagus* and the addition of 0.4% (w/v) glucose to the peptone culture also completely inhibited the synthesis of collagenase

[19]. On the other hand, 0.2% (w/v) glucose was used as carbon source for the production of extracellular collagenase by *B. pumilus* Col-J [20].

3.1.2 Nitrogen sources

Amongst the various organic nitrogen sources, Tryptone was found most suitable for the growth and production of collagenase (0.58 U/mL) by *Pseudomonas* sp (Fig. 2). Wu *et al*, (2010) reported tryptose as a nitrogen source which helps to produce maximum collagenase by *B. pumilus* Col-J [20]. Earlier, 0.5 % (w/v) tryptone was used for the optimum production of collagenase by *B. licheniformis* F11.4 [21]. Nitrogen source in the culture medium was found anessential component for the production of collagenase [22]. Moreover, peptone was also used for the production of collagenase, but casamino acids and various individual amino acids were found to inhibit the production of collagenase [23].

3.1.3 Growth supplements

Various growth supplements were added at a concentration of 0.2% (w/v) to the production medium (pH 6.5) (Fig. 3). The addition of 0.25% (w/v) yeast extract as growth supplement to the production medium gave maximum collagenase production (0.669 U/mL) by *Pseudomonas* sp. The same concentration of yeast extract (0.25%, w/v) was also reported earlier for the production of collagenase by *B. licheniformis* F11.4 [21]. The addition of yeast extract along with carbon and nitrogen sources in production medium gave comparatively better production of collagenase (0.604 U/mL) than the control. Similarly, the addition of yeast extract as growth supplement was reported to enhance the production of collagenase by the *B. subtilis* FS-2 and *Bacillus* sp. strain MO-1[24-25].

3.1.4 Optimization of additional growth

An increase in the collagenase activity (0.750 U/mL) was observed when meat extract was used in combination with yeast extract in the optimized production medium components (Fig. 4). It was also observed that the addition of meat extract to the yeast extract containing production medium enhances the production of collagenase by *Pseudomonas* sp. Therefore, the concentration of meat extract was also optimized to find out the appropriate concentration of meat extract for the maximum collagenase activity. The maximum production of collagenase (0.759 U/mL) was observed at 0.2% (w/v) concentration of meat extract in the production medium, additionally containing 0.25 % (w/v) yeast extract and other optimized medium components.

3.2 Factorial combinations (2^6) of optimized physicochemical parameters for the growth and production of collagenase by Pseudomonas sp.

The production medium (pH 6.5) containing (%, w/v) sucrose 1.25, tryptone 1.0, yeast extract 0.25, meat extract 0.2 and gelatin0.3 was used for the factorial design or technological combinations. In the current experiment, instead of one parameter being varied, the different combination of optimum and next nearest level of optimized parameters was used. In each case, growth, final pH and collagenase production by *Pseudomonas* sp. were monitored. Total 64 combinations (2^6) were obtainedby above parameters (Table 1). It was interesting that from all 64 factorial combinations, the maximum collagenase production (1.083 U/mL) was obtained with the combinations of physical and chemical parameters ($\mathbf{C_{57}}$) that includes medium (pH 7.0) containing (%, w/v) sucrose 1.0, tryptone 1.0, yeast extract 0.25 and meat extract 0.2; incubated at 37^{o}C.A full factorial design was also reported by Lima *et al*, (2009) to identify the effects and interactions of the initial medium pH, soybean flour concentration, temperature and orbital agitation speed on extracellular collagenase production by *Penicillium aurantiogriseum* URM4622 [26]. We observed that the 57th combination was ideal and optimized in all respects for the production of collagenase.

3.3 Effect of agitation speed on the growth and production of collagenase by Pseudomonas sp.

The effect of varying agitation speeds was studied on cell growth, production of collagenase and change in dissolved oxygen level by *Pseudomonas* sp. The increase in the agitation speed from 150 rpm to 300 rpm proved to be beneficial for the growth and production of collagenase by *Pseudomonas* sp. The maximum cell mass (2.82 mg/mL) of *Pseudomonas* sp. was obtained at 16th h of fermentation at 300 rpm, which was higher than the cell mass attained at 150 and 450 rpm (Fig. 5a). Further, the maximum cell mass at 150 and 300 rpm agitation was attained after 18th h and 16th h of cultivation, respectively. However, at higher agitation speed, the shearing forces also become operative and sometimes prove to be harmful both for growth as well as the production of collagenase by *Pseudomonas* sp. At 450 rpm the growth declined after 10th h and caused an early attainment of the stationary as well as death phase. The increase in agitation rate produces higher shear stress in the broth, which may cause a decrease in the growth of shear-sensitive microorganisms. The maximum collagenase activity (2.28 U/mL) was obtained after 16th h of cultivation at 300 rpm (Fig. 5b.). A further increase in fermentation time proved to be ineffective for the enhancement of the collagenase activity by *Pseudomonas* sp. The static decrease in collagenase production was observed after 16th h. However, at the higher speed (450 rpm), the effect of shearing forces becomes more prominent which result in decreased growth and enzyme production.

The dissolved oxygen profile of the fermentation broth under different agitation reveals that depletion in the dissolved oxygen was severe at the lower rate of agitation (Fig. 5c). The dissolved oxygen was declined from 100% (saturation) to 1.1% (saturation) during first 16^{th} h of the fermentation at an agitation rate of 150 and 300 rpm and remained constant throughout fermentation.

Further, the dissolved oxygen level at higher agitation rate (450 rpm) dropped rapidly below 9% (saturation) during first six hours and then started increasing from 16^{th} h onwards and reached to 93% at 22^{th} h of fermentation. It has been found that low level of dissolved oxygen results in increased cell growth and collagenase production by *Pseudomonas* sp. with better utilization of oxygen for the physiochemical and metabolic activity of cell. For an optimal enzyme production, it seems to be necessary to reach a good mix of the culture broth since agitation produces a dispersion of air in the culture medium, homogenizes the temperature and the pH improves transference rate of nutrients. However, high speeds of agitation act against the enzymatic activity, probably due to the shear stress caused by the blade tips of the impeller, which increase as the revolution speed increases [27]. Stress condition may contribute negatively toward cell growth and enzyme stability.

3.4 Effect of aeration rate on growth and production of collagenase by Pseudomonas sp.

The optimization of different aeration rates (0.25, 0.5 and 0.75 vvm) was carried out for the collagenase production by *Pseudomonas* sp., constant agitation speed (300 rpm) in a 14 L fermenter (6 L working volume) and its effect on the growth and collagenase production was studied up to 24 h of fermentation. The growth of *Pseudomonas* sp. was greatly affected by the supply of oxygen during the course of fermentation. The maximum growth (3.73 mg/mL) of *Pseudomonas* sp. was obtained at 16^{th} h of fermentation at 0.5vvm (aeration rate) and 300 rpm agitation speed (Fig. 6a). The maximum collagenase production by *Pseudomonas* sp. (2.52 U/mL) was observed at 14^{th} h of fermentation at 0.5 vvm aeration followed by 2.37 U/mL at 12^{th} h (Fig. 6b). These results suggest that air flow rate of 0.5 vvm not only favoured maximal cell growth but also enhanced collagenase production. However, there was decrease in collagenase activity in case of *Pseudomonas* sp. with increase in aeration rate from 0.50 vvm to 0.75 vvm. This might be due to the inhibitory effect of the high dissolved oxygen concentration during the course of cultivation. The dissolved oxygen concentration reduced drastically during 2-10 h of fermentation because the growing cells of *Pseudomonas* sp. utilized the oxygen rapidly for their own physiological activity. However, at 0.5 and 0.75 vvmaeration the dissolved oxygen level increased rapidly after 16^{th} h of incubation (Fig. 6c).

3.5 Course of cultivation of Pseudomonas sp. in a laboratory scale fermenter

Course of cultivation of *Pseudomonas* sp. and production of collagenase without control of pH has been studied at laboratory scale fermenter with the vessel of capacity 14 L (6 L working volume) at 37^{o}C. The production medium (pH 7.0) containing (%, w/v) sucrose 1.0; tryptone 1.0; yeast extract 0.25; meat extract 0.20 and gelatin 0.3 was inoculated with 21 h old seed culture (4 %, v/v) at the agitation speed of 300 rpm and 0.50 vvm aeration rate. Samples were taken at interval of 2 h and analysed for DO, final pH, cell growth and collagenase activity (Fig. 7). Dissolved oxygen profile showed decline from 100% to 1.2% at 10th h and again started to rise after 14th h and then reached upto 100%. The rapid decrease in dissolved oxygen level was found to be associated with microbial growth. The pH profile showed that neutral pH favours cell growth and enzyme production but pH slightly moves towards alkalinity. Maximum cell growth (3.73 mg/mL) observed at 16th h of incubation and thereafter a slight decline in cell mass content was seen. Maximum enzyme activity (2.52 U/mL) observed at 14th h of fermentation and afterwards a constant decrease in enzyme activity was observed. The bench-scale production of extracellular collagenase from *Pseudomonas* sp. carried out at 300 rpm agitation and 0.5 vvm aeration rate in 6 L production medium in laboratory scale fermenter led to 2.3-fold increase in collagenase activity as well as reduction in time of cultivation (14th h) compared to shake flask (24th h).

4.0 Conclusion

Protein wastes in Himalayan region are abundantly found in or near meat and fish market. The soil/sewage samples collected from the local fish market and slaughter house are screened for collegenase activity. The collagenase was isolated, purified and characterized by our group and further factorial design was used for the up scaling of collagenase production. All the physiochemical parameters were successfully optimized. Therefore, a factorial design on the basis of optimized parameters have been developed for the bench-scale production ofcollagenase from *Pseudomonas* sp. The bench-scale fermenter led to a 2.3-fold increase in collagenase activity with a reduction in time of cultivation (14th h) compared to shake flask (24th h).

Conflict of interest

Authors have no conflict of interest

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329 **Table 1.** Technological combinations of optimized physicochemical parameters for
330 growth and collagenase production by *Pseudomonas* sp.

331 **Table 1**

332

S. No.	Initial pH	T* (^{o}C)	Sucrose (%, w/v)	Tryptone (%, w/v)	Yeast extract (%, w/v)	Meat extract (%, w/v)	Cell mass (mg/mL)	Enzyme activity (U/mL)	Final pH
1.	6.5	30	0.75	1.00	0.25	0.20	1.76	0.675	8.49
2.	6.5	30	0.75	1.00	0.25	0.30	1.39	0.420	8.44
3.	6.5	30	0.75	1.00	0.30	0.20	1.56	0.410	8.49
4.	6.5	30	0.75	1.00	0.30	0.30	2.13	0.769	8.54
5.	6.5	30	0.75	1.25	0.25	0.20	1.68	0.533	8.33
6.	6.5	30	0.75	1.25	0.25	0.30	2.09	0.432	8.37
7.	6.5	30	0.75	1.25	0.30	0.20	2.13	0.437	8.43
8.	6.5	30	0.75	1.25	0.30	0.30	1.68	0.352	8.42
9.	6.5	30	1.00	1.00	0.25	0.20	2.09	0.698	8.45
10.	6.5	30	1.00	1.00	0.25	0.30	2.13	0.452	8.40
11.	6.5	30	1.00	1.00	0.30	0.20	2.01	0.446	8.37
12.	6.5	30	1.00	1.00	0.30	0.30	1.76	0.936	8.49
13.	6.5	30	1.00	1.25	0.25	0.20	2.09	0.769	8.44
14.	6.5	30	1.00	1.25	0.25	0.30	1.60	0.668	8.46
15.	6.5	30	1.00	1.25	0.30	0.20	2.13	0.383	8.38
16.	6.5	30	1.00	1.25	0.30	0.30	1.76	0.579	8.56
17.	6.5	37	0.75	1.00	0.25	0.20	1.89	0.967	8.62
18	6.5	37	0.75	1.00	0.25	0.30	2.21	0.984	8.56
19.	6.5	37	0.75	1.00	0.30	0.20	1.80	0.468	8.67
20.	6.5	37	0.75	1.00	0.30	0.30	1.76	0.348	8.58
21.	6.5	37	0.75	1.25	0.25	0.20	1.80	0.720	8.53
22.	6.5	37	0.75	1.25	0.25	0.30	1.76	0.345	8.61
23.	6.5	37	0.75	1.25	0.30	0.20	1.80	0.357	8.71
24.	6.5	37	0.75	1.25	0.30	0.30	2.17	0.380	8.80
25.	6.5	37	1.00	1.00	0.25	0.20	1.60	0.475	8.58
26.	6.5	37	1.00	1.00	0.25	0.30	1.56	0.274	8.70
27.	6.5	37	1.00	1.00	0.30	0.20	1.85	0.174	8.67
28.	6.5	37	1.00	1.00	0.30	0.30	2.26	0.567	8.80
29.	6.5	37	1.00	1.25	0.25	0.20	2.13	0.715	8.70
30	6.5	37	1.00	1.25	0.25	0.30	2.34	0.393	8.68
31.	6.5	37	1.00	1.25	0.30	0.20	2.42	0.642	8.73
32.	6.5	37	1.00	1.25	0.30	0.30	2.18	0.773	8.68
33.	7.0	30	0.75	1.00	0.25	0.20	1.80	0.825	8.60
34.	7.0	30	0.75	1.00	0.25	0.30	1.89	0.377	8.57
35.	7.0	30	0.75	1.00	0.30	0.20	1.97	0.644	8.64
36.	7.0	30	0.75	1.00	0.30	0.30	2.01	0.323	8.59
37.	7.0	30	0.75	1.25	0.25	0.20	1.60	0.522	8.32
38.	7.0	30	0.75	1.25	0.25	0.30	1.72	0.411	8.58

39.	7.0	30	0.75	1.25	0.30	0.20	2.05	0.449	8.53
40.	7.0	30	0.75	1.25	0.30	0.30	2.42	0.535	8.59
41.	7.0	30	1.00	1.00	0.25	0.20	2.13	0.649	8.60
42.	7.0	30	1.00	1.00	0.25	0.30	2.34	0.332	8.54
43.	7.0	30	1.00	1.00	0.30	0.20	2.58	0.436	8.55
44.	7.0	30	1.00	1.00	0.30	0.30	2.13	0.339	8.56
45.	7.0	30	1.00	1.25	0.25	0.20	1.85	0.447	8.65
46.	7.0	30	1.00	1.25	0.25	0.30	1.68	0.686	8.53
47.	7.0	30	1.00	1.25	0.30	0.20	2.13	0.770	8.44
48.	7.0	30	1.00	1.25	0.30	0.30	2.54	0.287	8.62
49.	7.0	37	0.75	1.00	0.25	0.20	1.84	0.686	8.67
50.	7.0	37	0.75	1.00	0.25	0.30	2.64	0.521	8.76
51	7.0	37	0.75	1.00	0.30	0.20	3.65	0.712	8.83
52.	7.0	37	0.75	1.00	0.30	0.30	2.50	0.418	8.79
53.	7.0	37	0.75	1.25	0.25	0.20	3.08	0.741	8.87
54.	7.0	37	0.75	1.25	0.25	0.30	3.03	0.603	8.74
55.	7.0	37	0.75	1.25	0.30	0.20	2.54	0.667	8.78
56.	7.0	37	0.75	1.25	0.30	0.30	2.30	0.329	8.91
57.	**7.0**	**37**	**1.00**	**1.00**	**0.25**	**0.20**	**2.71**	**1.083**	8.78
58.	7.0	37	1.00	1.00	0.25	0.30	2.42	0.459	8.72
59.	7.0	37	1.00	1.00	0.30	0.20	2.87	0.546	8.82
60.	7.0	37	1.00	1.00	0.30	0.30	2.05	0.456	8.74
61.	7.0	37	1.00	1.25	0.25	0.20	2.46	0.658	8.86
62.	7.0	30	1.00	1.25	0.25	0.30	2.42	1.076	8.69
63	7.0	30	1.00	1.25	0.30	0.20	2.50	0.559	8.73
64.	7.0	30	1.00	1.25	0.30	0.30	2.58	0.491	8.72

T* (^{o}C) = Temperature (^{o}C)

Figures

Fig.1. Optimization of various carbon sources for the production of extracellular collagenase by *Pseudomonas* sp.

Fig. 2. Optimization of various nitrogen sources for the production of extracellular collagenase by *Pseudomonas* sp.

Fig. 3. Optimization of various growth supplements for the production of extracellular collagenase by *Pseudomonas* sp.

Fig.4. Optimization of additional growth supplements for the production of extracellular collagenase by *Pseudomonas* sp.

Fig. 5 (a). Effect of agitation speed on growth of *Pseudomonas* sp.

Fig. 5(b). Effect of agitation speed on production of collagenase by *Pseudomonas* sp.

Fig. 5(c). Effect of agitation speed on dissolved oxygenof fermentation broth of *Pseudomonas* sp.

Fig. 6(a). Effect of areation rate on growth of *Pseudomonas* sp.

Fig. 6(b). Effect of areation rate on collagenase production by *Pseudomonas* sp.

Fig. 6(c). Effect of areation rate on dissolced oxygen of fermentation broth of *Pseudomonas* sp.

Fig. 7. Course of fermentation of *Pseudomonas* sp.

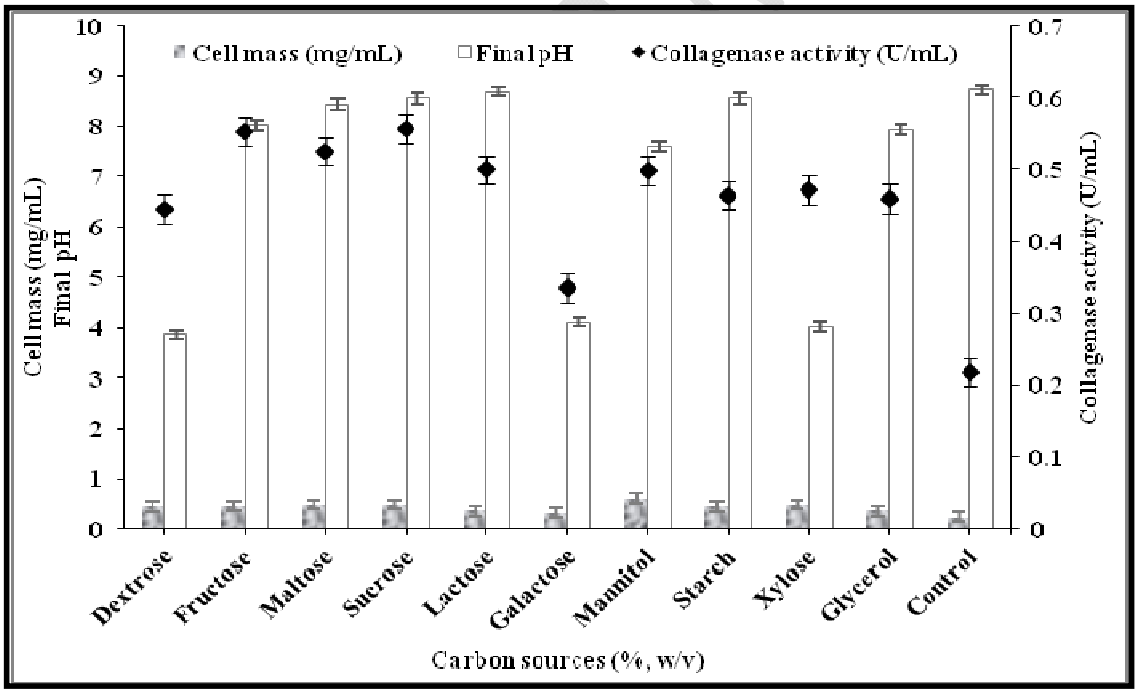
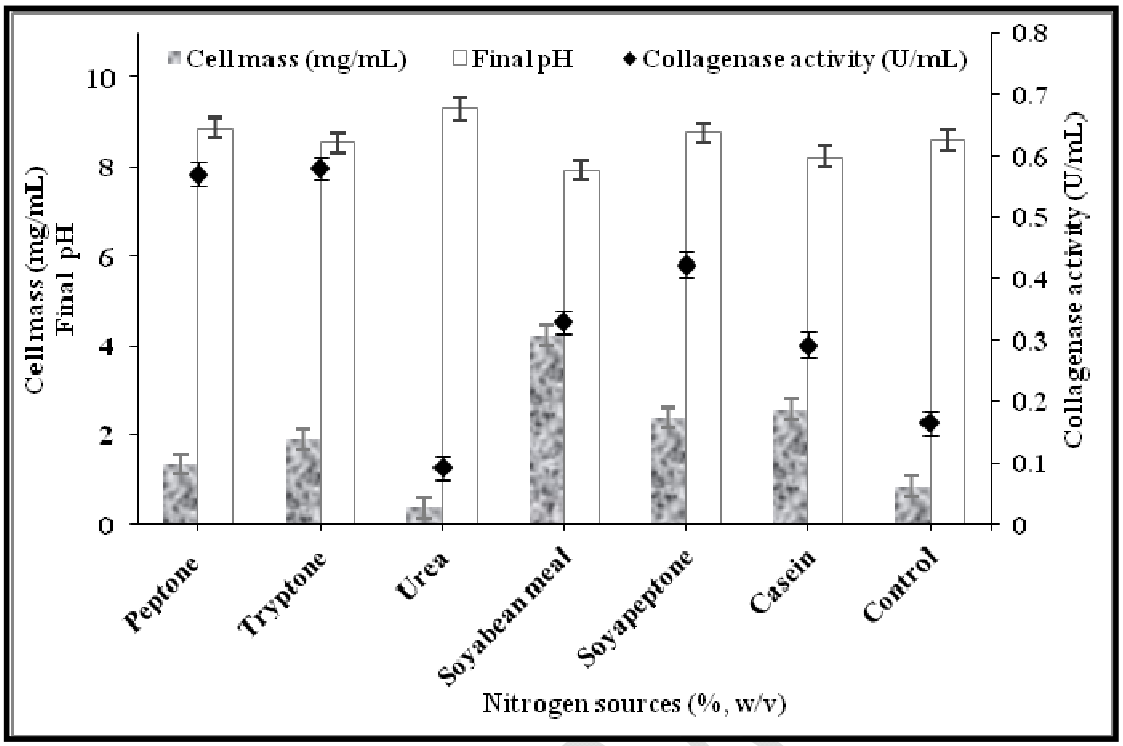
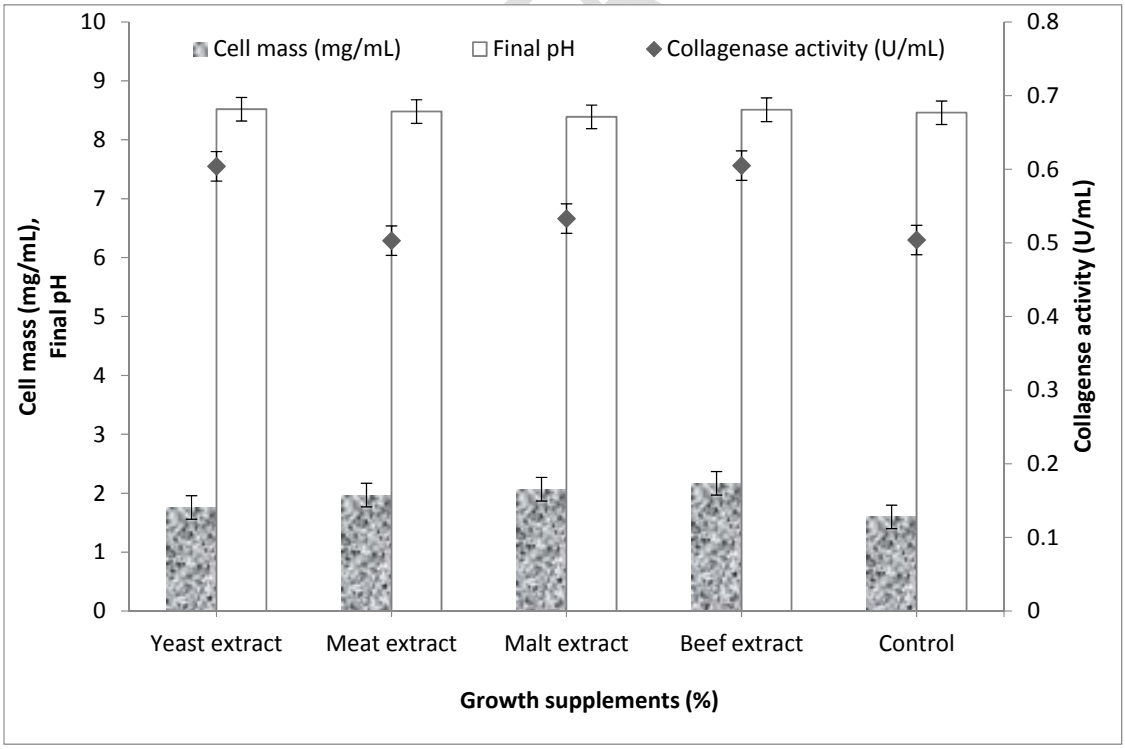


Fig. 1



370

371 **Fig. 2**



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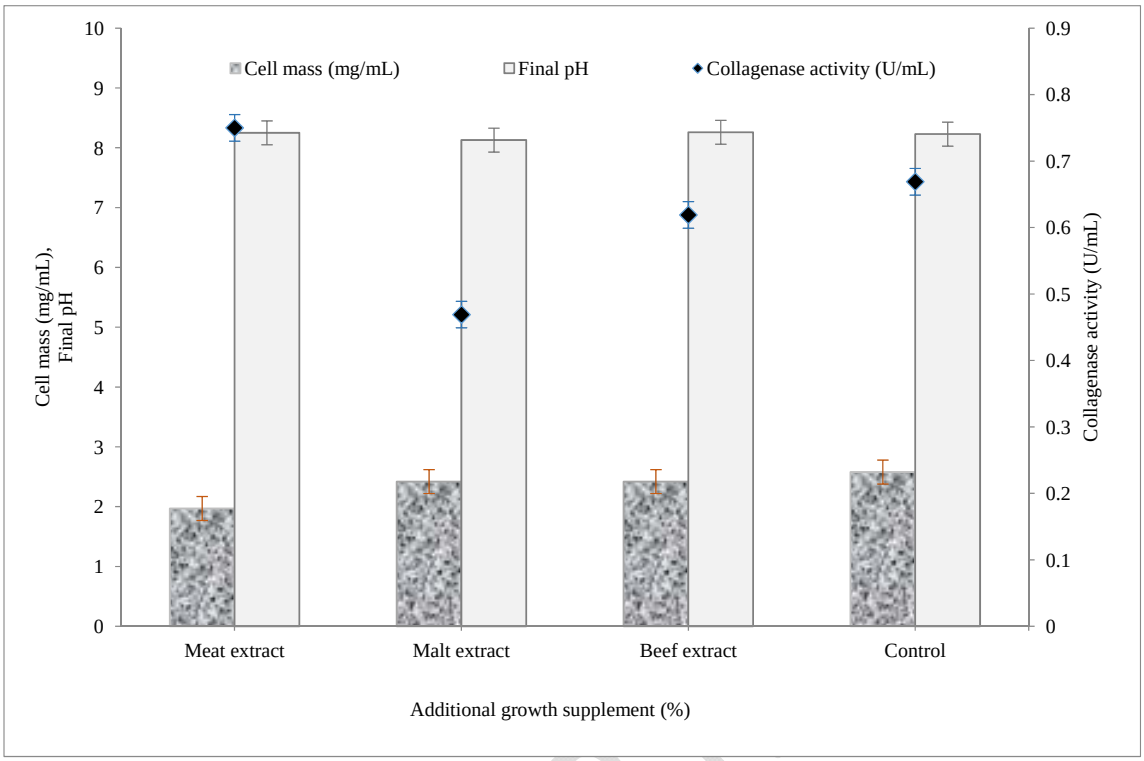
373 **Fig. 3**



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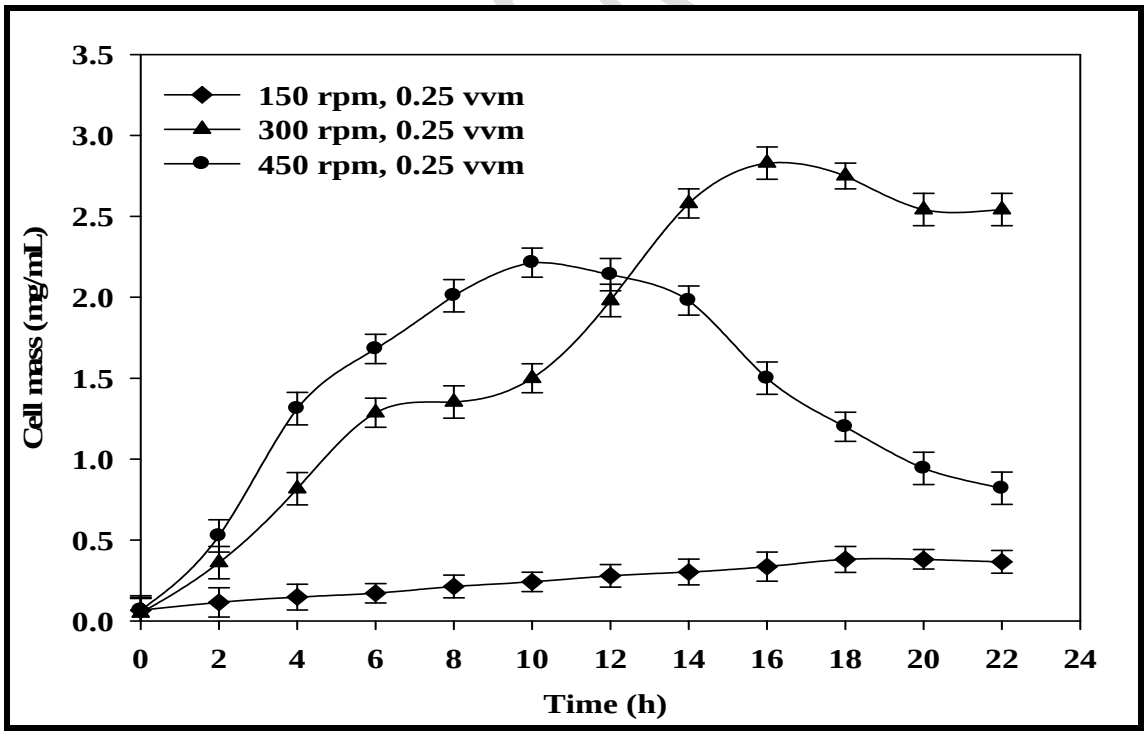
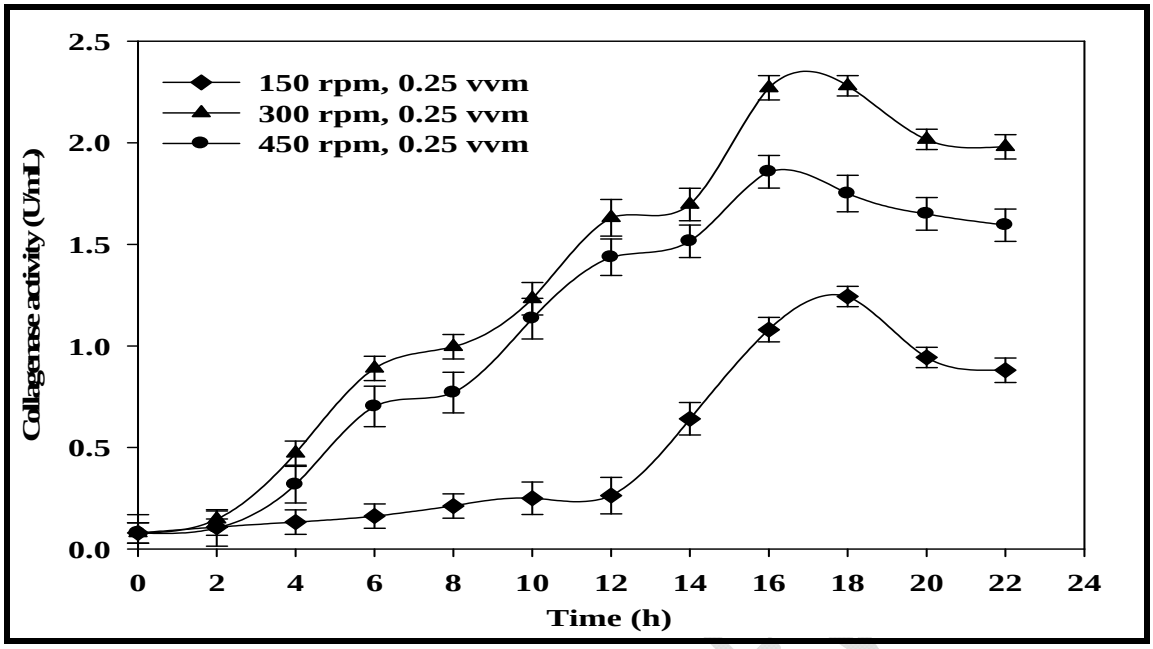
375 **Fig. 4**



376
377

378 **Fig. 5 (a)**



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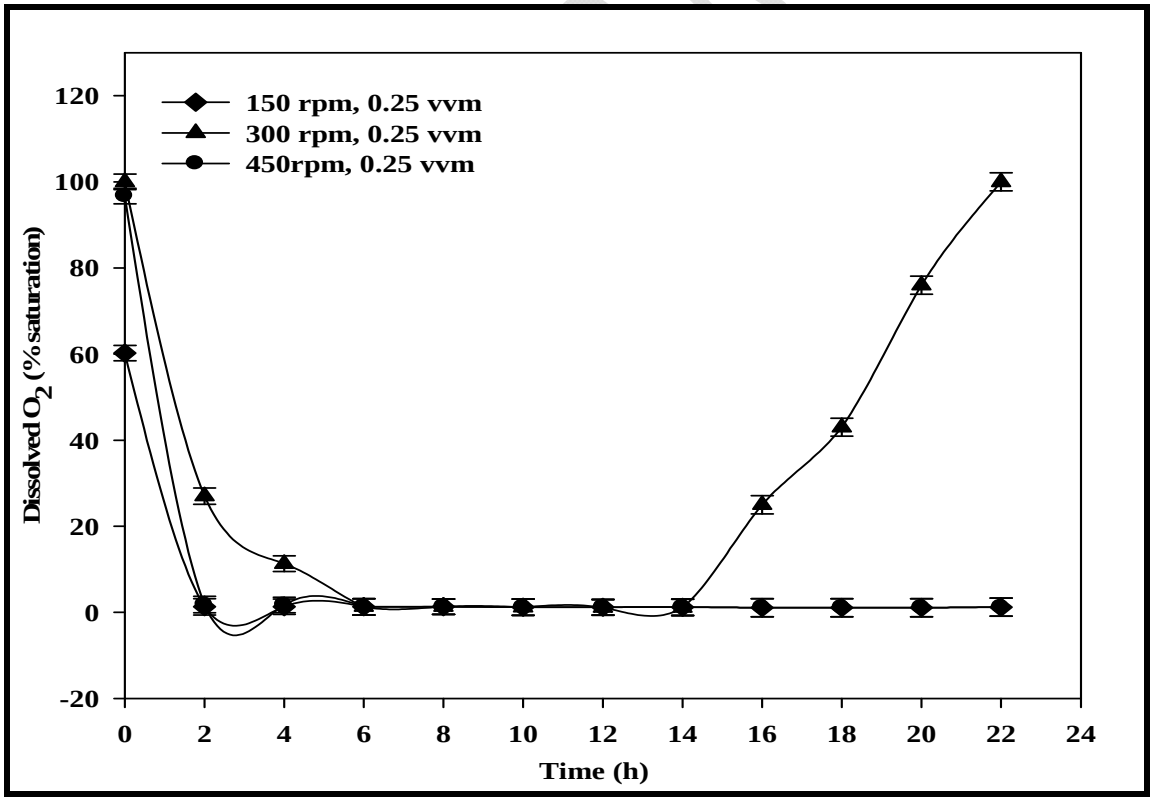
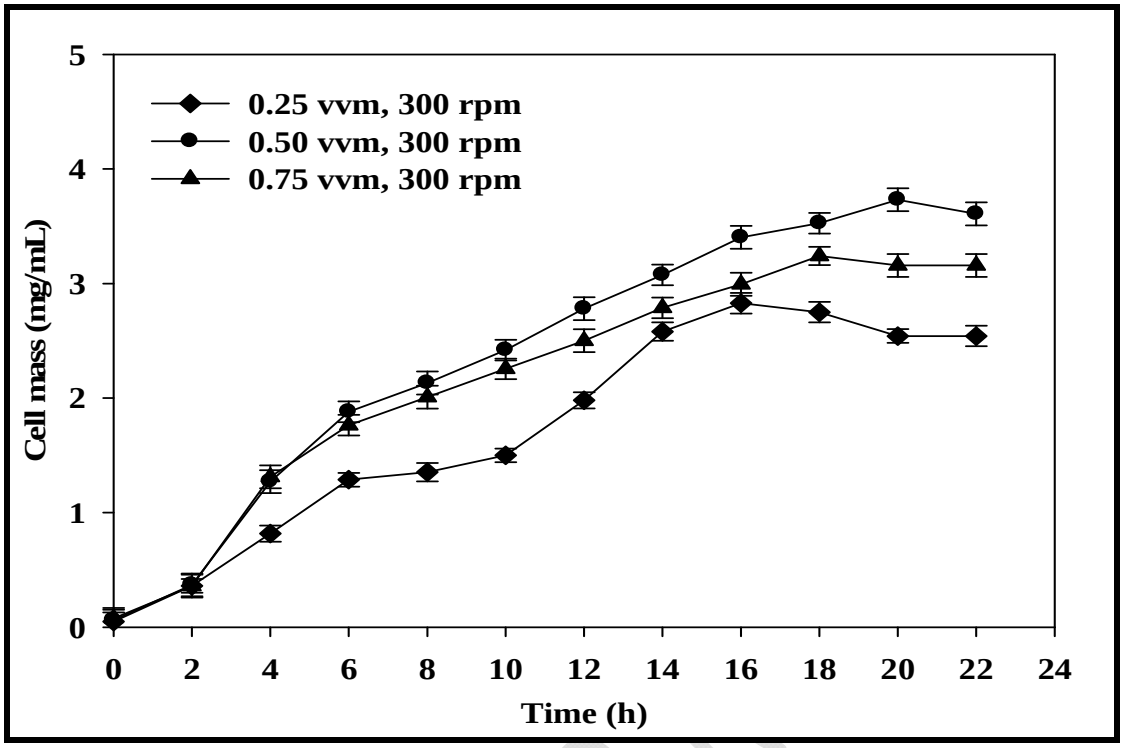
381 **Fig. 5 (b)**



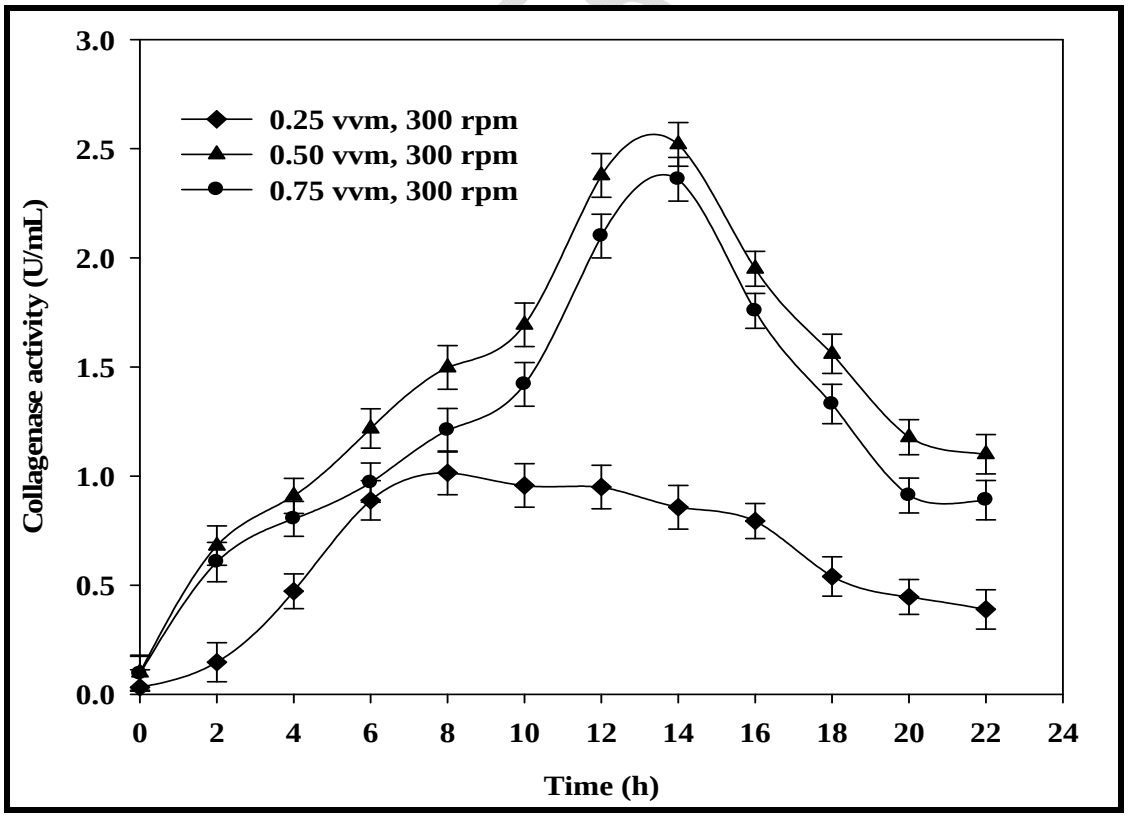
382
383

384 **Fig. 5 (C)**



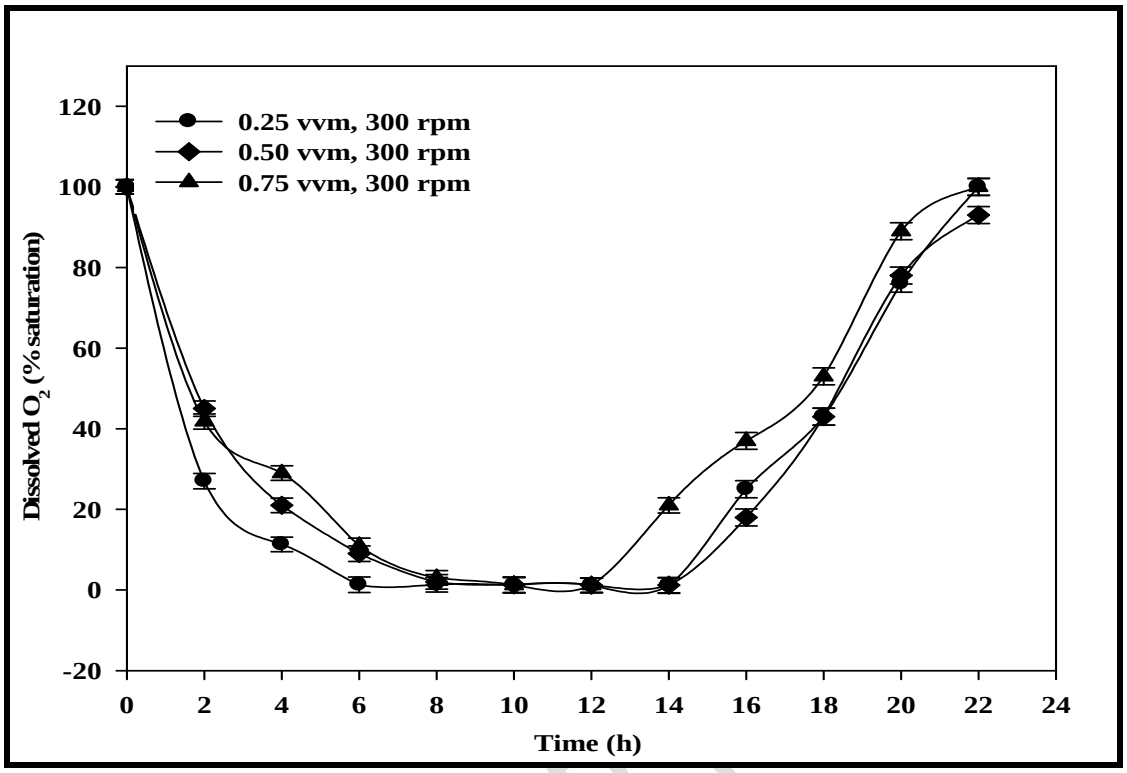
385

386 **Fig. 6 (a)**



387

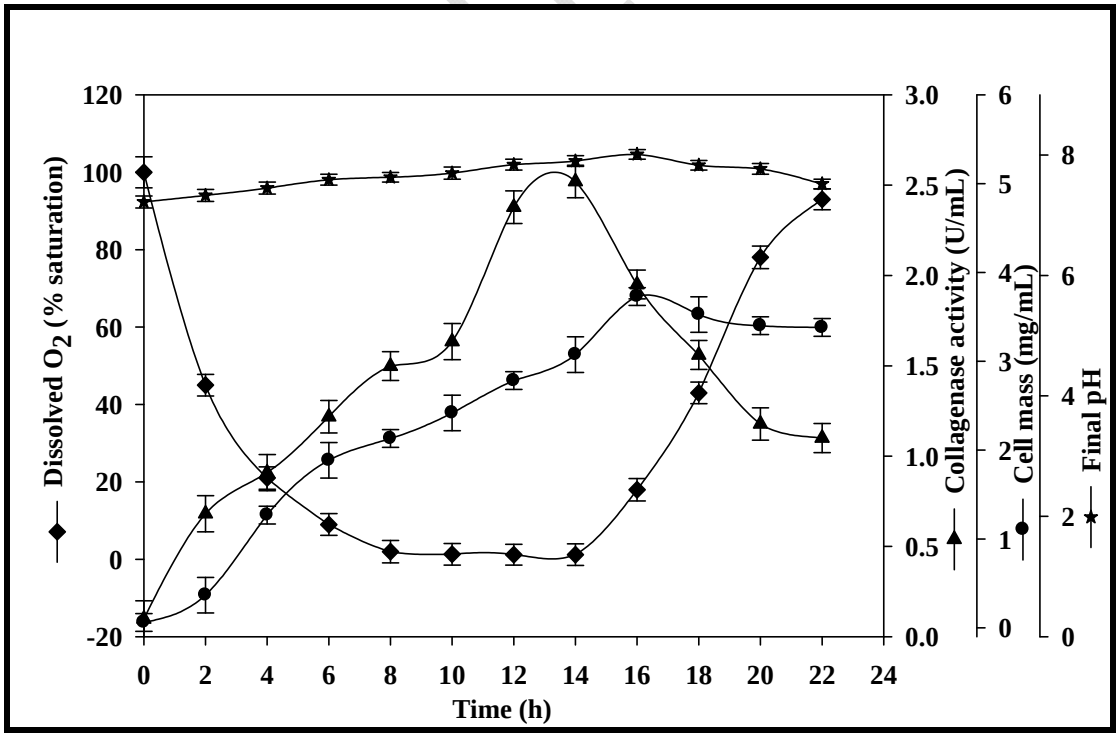
388 **Fig. 6 (b)**



389

390 **Fig. 6 (C)**

391



392

393 **Fig. 7**