Original Research Article

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

Abstract

The current study was focused on factorial design based bench-scale production of collagenase by *Pseudomonas* sp. Chemical and fermentation conditions including medium components (carbon, nitrogen, and growth supplements) were optimized. The medium containing sucrose, tryptone and gelatin substrate was found to enhance the production of collagenase. The physical parameters (agitation speed and aeration rate) were also optimized. Moreover, the interactive effect of optimized physicochemical parameters using two levels of six factors (2^6) of factorial design was studied for the maximum collagenase production. Among 64 combinations, the 57th combination was shown maximum 1.43 U/mL collagenase activity. The bench-scale production of collagenase was achieved in a 6 L working volume laboratory fermenter. The bench-scale fermenter produced 2.3-fold enhanced collagenase activity at reduced cultivation time (14th h) in comparison to the shake flask (24th h). The lead combination can be used for the large scale collagenase production in industerial fermenters.

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 a 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 remodeling 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]. Recently, screening, isolation, characterization, and purification of collagenase from *Pseudomonas* sp. found in protein waste of Himalayan region was reported [16-17]. Whereas, Sayak Bhattacharya [18], reported the role of novel collagenase in degradation of the skeletal fibers of great barrier reef sponge (*Rhopaloeides odorabile*,) which require Ca^{2+} and Zn^{2+} as cofactors. *Clostridium histolyticum* collagenase used for therapeutic purpose (Peyronie's disease) from 19th century [19]. In addition, collagenase produced from *Grimontia hollisae*strain 1706B (gram negative) resulted in better collagen hydrolysis than that of produced from a gram-positive *Clostridium histolyticum* [20]. Collagenase produced from *Pseudomonas sp.*, also reported for fish and plant root-knot nematode (*Meloidogyne javanica*) digestive property [21]. Earlier literature reported that physicochemical conditions significantly influenced the yield of extracellular enzymes. Therefore, optimization of parameters for the bench-scale production of collagenase by *Pseudomonas* sp. is required. Thus, an attempt has been made to use the combinational effect of physical and chemical factors using permutation and combination to produce a maximum amount of collagenase at fermentor scale.

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 the maximum amount of collagenase by *Pseudomonas* sp.

2.1 Optimization for the bench scale production of collagenase by Pseudomonas sp.

In order to check the role of individual component of 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 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 production medium were used to check their effect on the growth and production of collagenase.

2.1.2 Nitrogen sources

Organic nitrogen sources (peptone, tryptone, urea, soybean meal extract, soyapeptone, and casein) were used for the growth and production of collagenase 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, 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. The interactive effect of optimized physicochemical parameters was studied using two levels of six factors (2^6), named as technological combinations/factorial design. 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 Collagenase production by Pseudomonas sp. in 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 also observed.

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

2.4.1 Agitation speed

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 analyzed 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.

2.4.2 Aeration rate

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.

2.5 Course of cultivation for Pseudomonas sp.

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 cultivation of *Pseudomonas* sp. was observed up to 24 h.

3.0 Results and Discussion

3.1 Optimization of parameters for the production of 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, [22] reported that the 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 completely inhibited the synthesis of collagenase [23]. On the other hand, 0.2% (w/v) glucose was used as a carbon source for the production of extracellular collagenase by *B. pumilus* Col-J [24].

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*. [24], reported tryptone as a nitrogen source, which helps to produce maximum collagenase by *B. pumilus* Col-J [24]. Earlier, 0.5 % (w/v) tryptone was used for the optimum production of collagenase by *B. licheniformis* F11.4 [25]. Nitrogen source in the culture medium was found an essential component for the production of collagenase [26]. 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 [27].

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 [25]. The addition of yeast extract along with carbon and nitrogen sources in the production medium gave comparatively better production of collagenase (0.604 U/mL) than the control. Similarly, the addition of yeast extract as a growth supplement was reported to enhance the production of collagenase by the *B. subtilis* FS-2 and *Bacillus* sp. strain MO-1[27-28].

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 obtained by 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) for the production of extracellular collagenase by *Penicillium aurantiogriseum* URM4622 [29]. The 57th combination was found ideal and optimized in all respects for the production of collagenase.

3.3 Effect of agitation speed and aeration rate collagenase production by Pseudomonas sp.

3.3.1 Agitation speed

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 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 16th 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 16th h onwards and reached to 93% at 22th 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 a cell. For 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 [30]. Stress condition may contribute negatively toward cell growth and enzyme stability.

3.3.2 Aeration rate

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. 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 16th h of fermentation at 0.5vvm (aeration rate) and 300 rpm agitation speed (Fig. 6a). Maximum collagenase production by *Pseudomonas* sp. (2.52 U/mL) was observed at 14th h of fermentation at 0.5 vvm aeration followed by 2.37 U/mL at 12th h (Fig. 6b). These results suggest that an airflow rate of 0.5 vvm not only favored maximal cell growth but also enhanced collagenase production. However, there was a

decrease in collagenase activity in case of *Pseudomonas* sp. with an 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 vvm aeration the dissolved oxygen level increased rapidly after 16^{th} h of incubation (Fig. 6c).

3.4 Course of cultivation for Pseudomonas sp. in a laboratory scale fermenter

The course of cultivation for *Pseudomonas* sp. and collagenase production 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 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 an interval of 2 h and analyzed for DO, final pH, cell growth and collagenase activity (Fig. 7). Dissolved oxygen profile showed a decline from 100% to 1.2% at 10^{th} h and again started to rise after 14^{th} h and then reached up to 100%. The rapid decrease in dissolved oxygen level was found to be associated with microbial growth. The pH profile showed that neutral pH favors cell growth and enzyme production but pH slightly moves towards alkalinity. Maximum cell growth (3.73 mg/mL) observed at 16^{th} h of incubation and thereafter a slight decline in cell mass content was seen. Maximum enzyme activity (2.52 U/mL) observed at 14^{th} h of fermentation and afterwards, a constant decrease in enzyme activity was observed. The bench-scale production of extracellular collagenase from *Pseudomonas* sp. was carried out at 300 rpm agitation and 0.5 vvm aeration rate in 6 L production medium in laboratory scale fermenter, led to a 2.3-fold increase in collagenase activity as well as a reduction in time of cultivation (14^{th} h) in comparison to shake flask (24^{th} h).

4.0 Conclusion

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

Conflict of interest

Authors have no conflict of interest

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

362 **Table 1**

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 the growth of *Pseudomonas* sp.

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

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

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

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

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

Fig. 7. The course of fermentation of *Pseudomonas* sp.

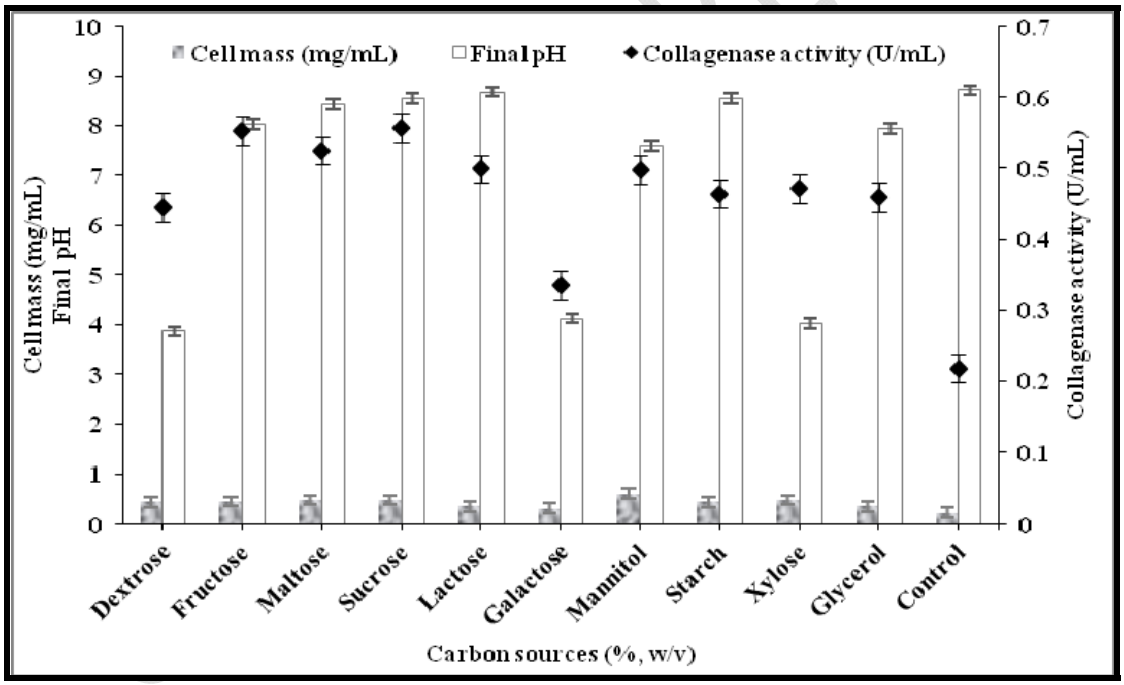
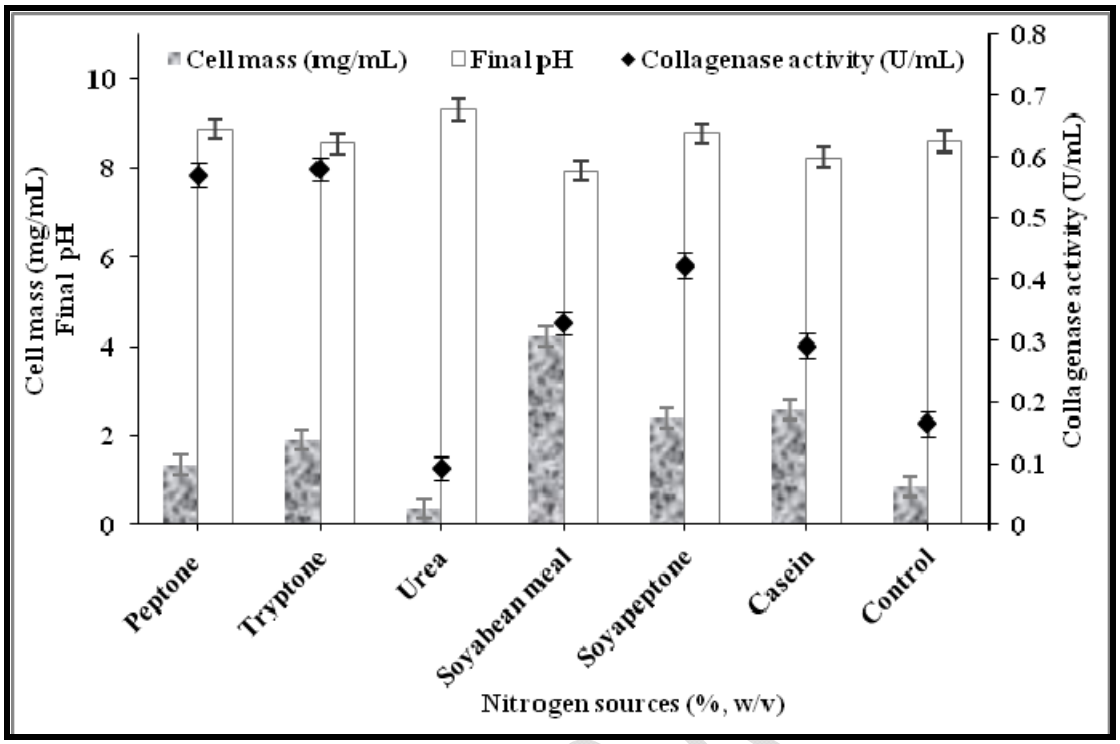
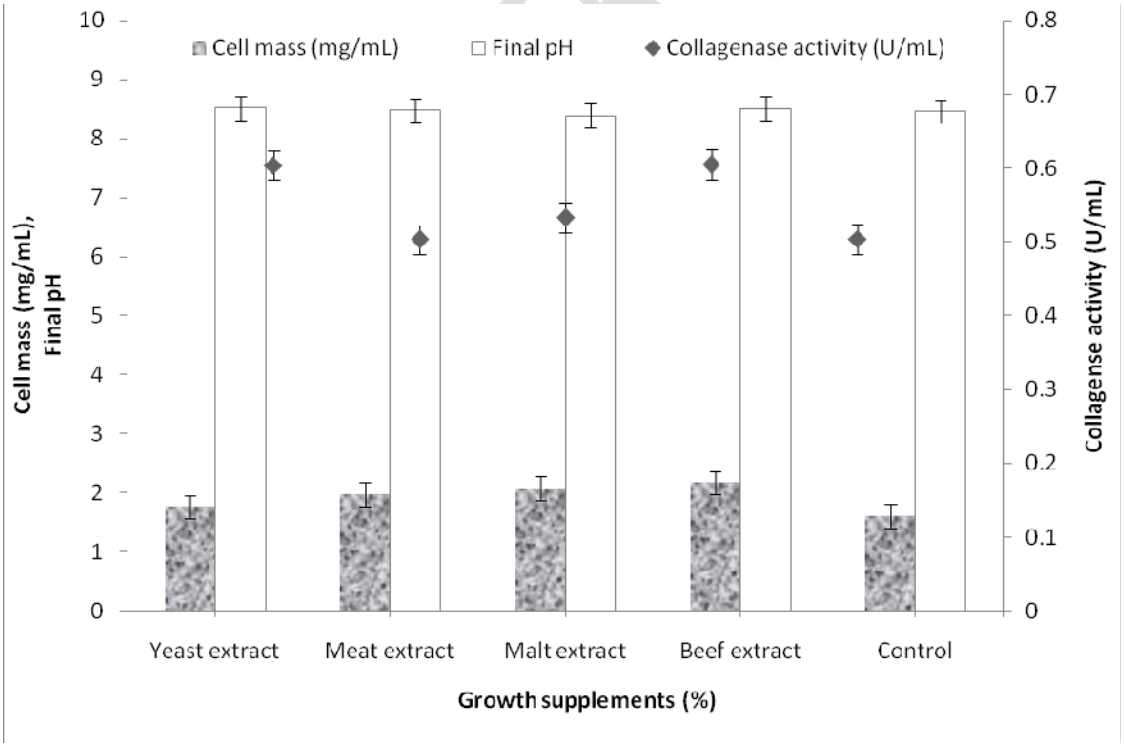


Fig. 1



400

401 **Fig. 2**



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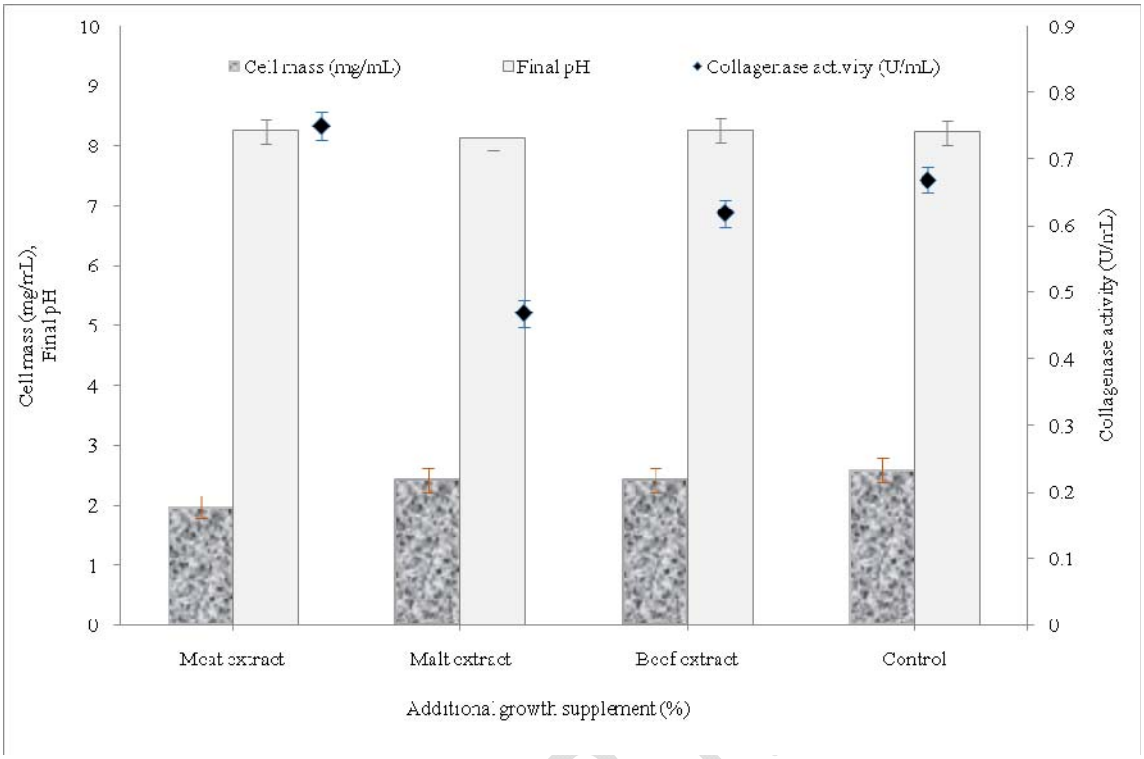
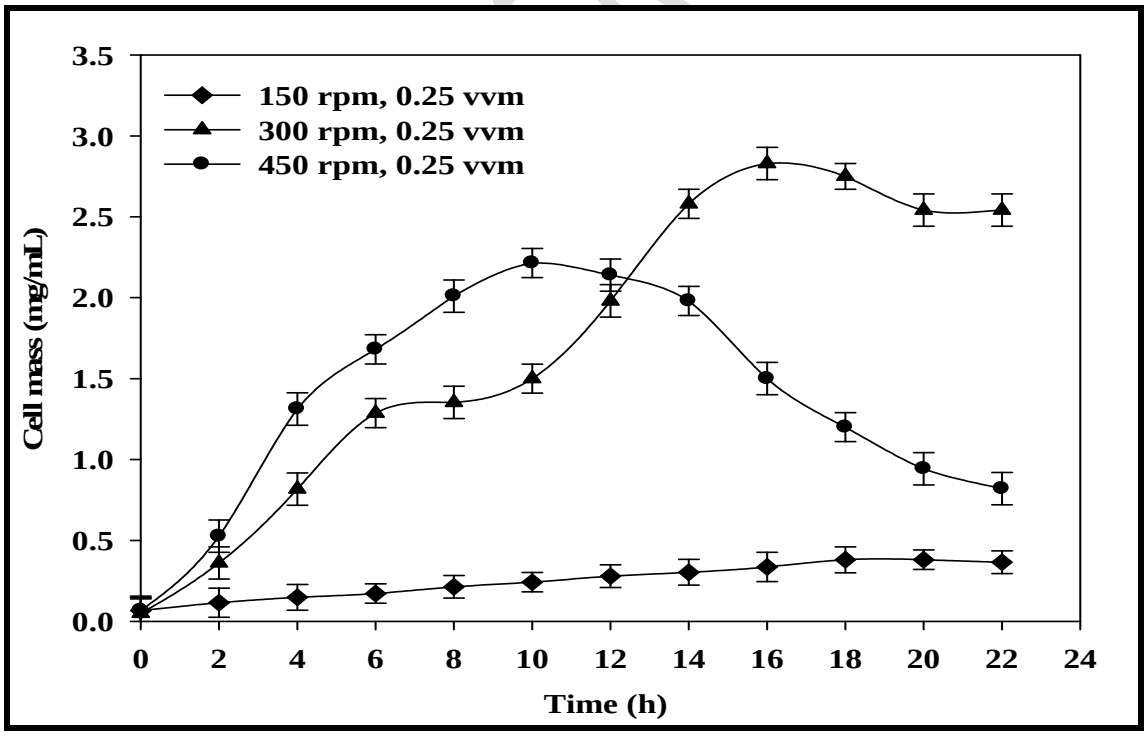
403 **Fig. 3**



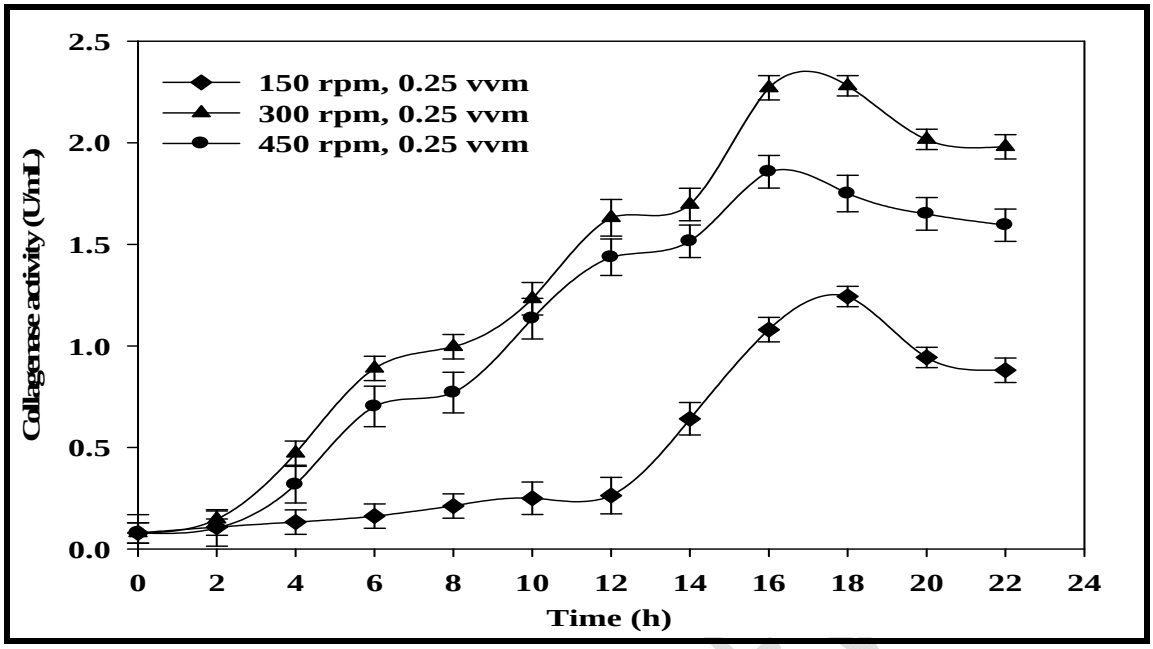
404

405 **Fig. 4**



406
407

408 **Fig. 5 (a)**



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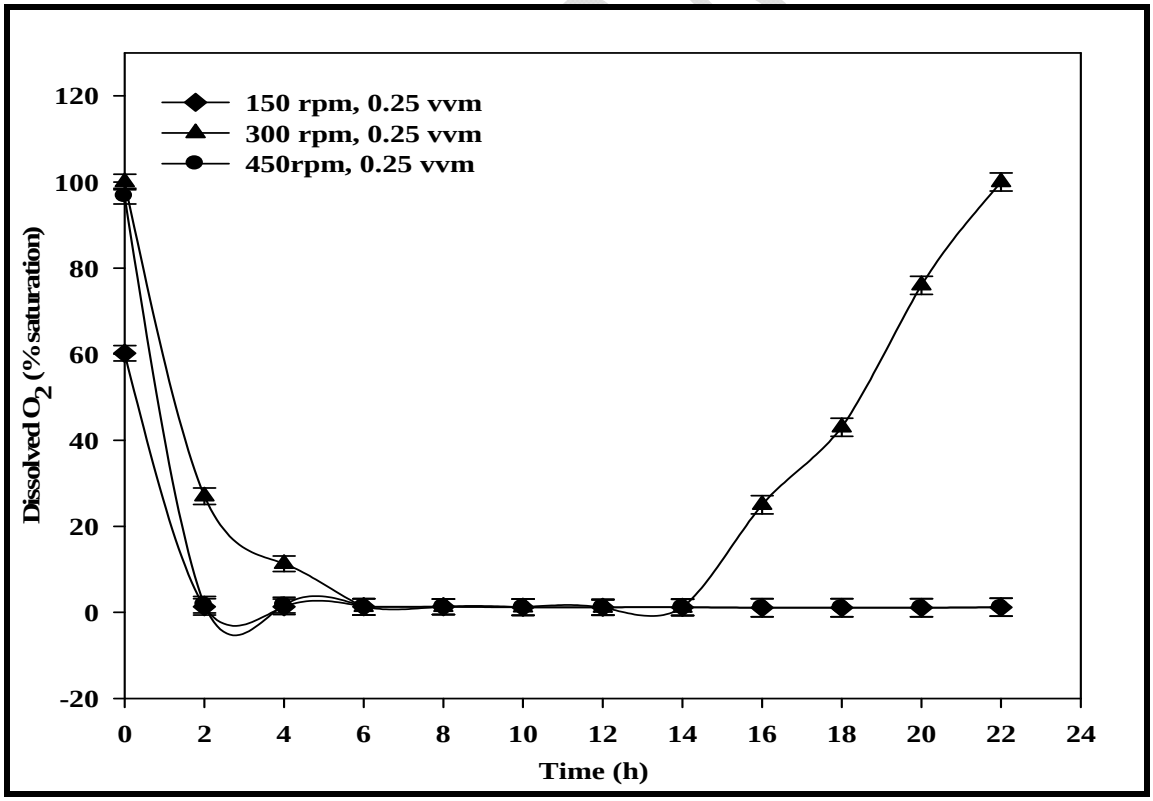
411 **Fig. 5 (b)**



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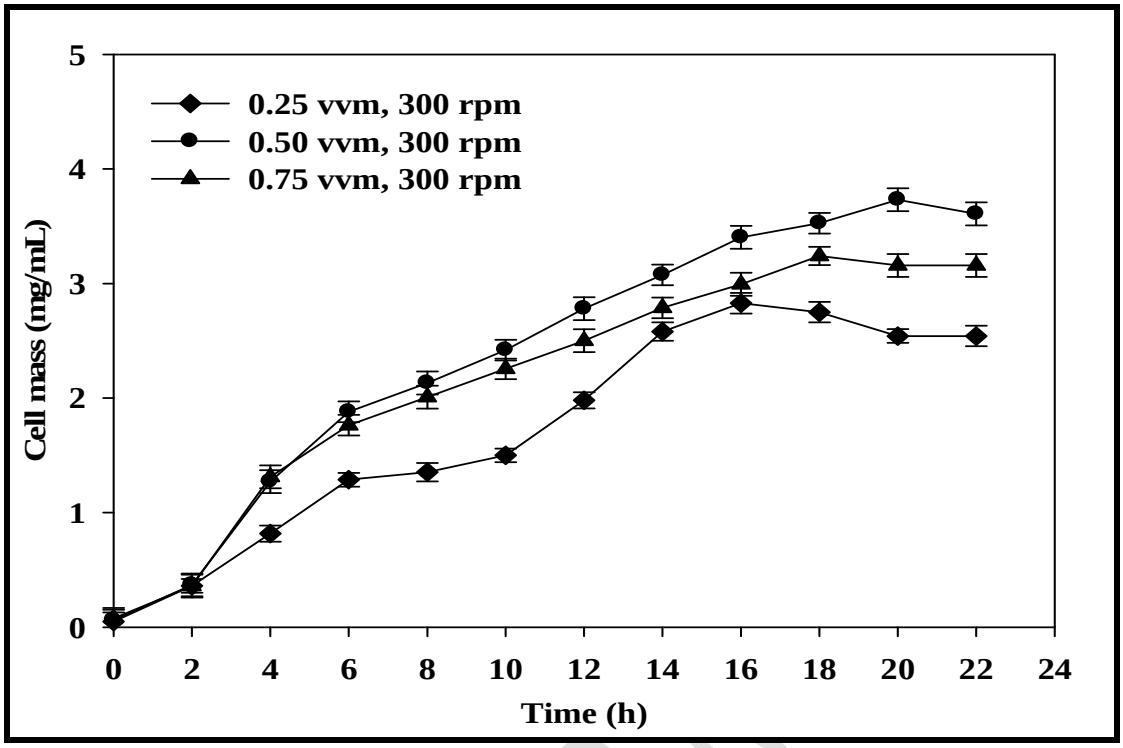
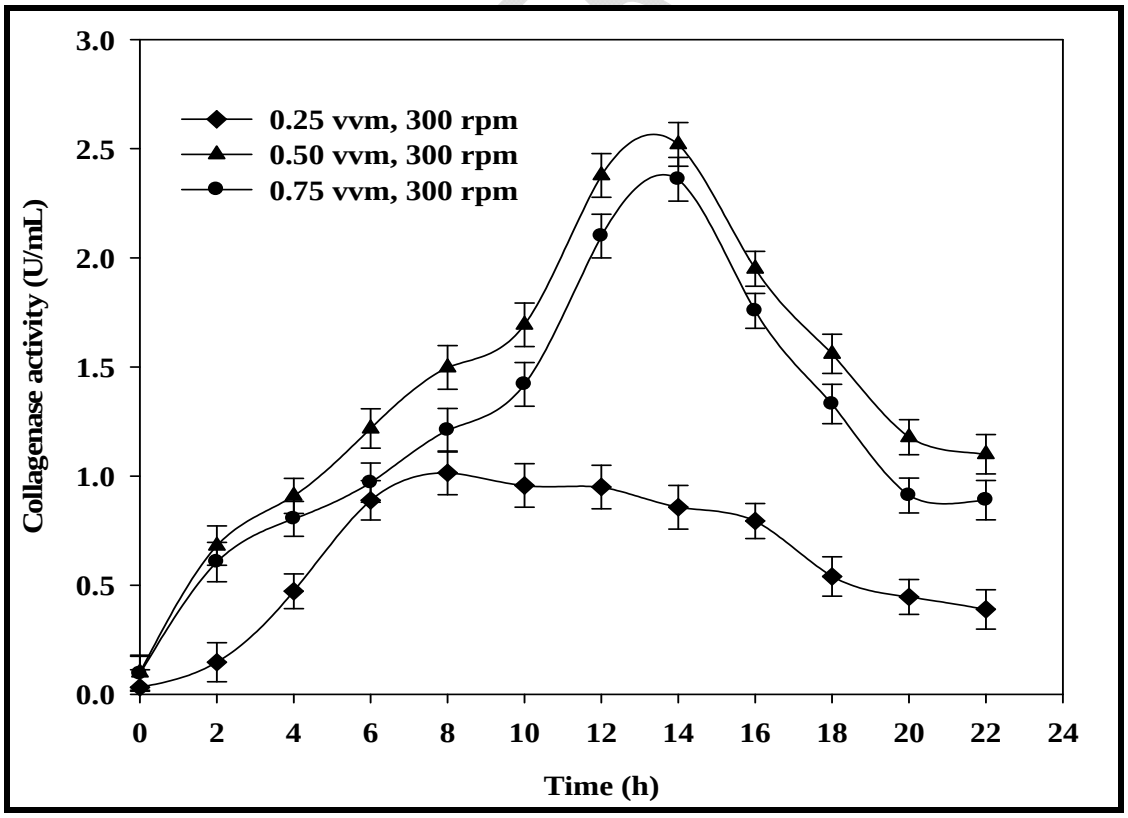
414 **Fig. 5 (C)**



415

416 **Fig. 6 (a)**



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418 **Fig. 6 (b)**

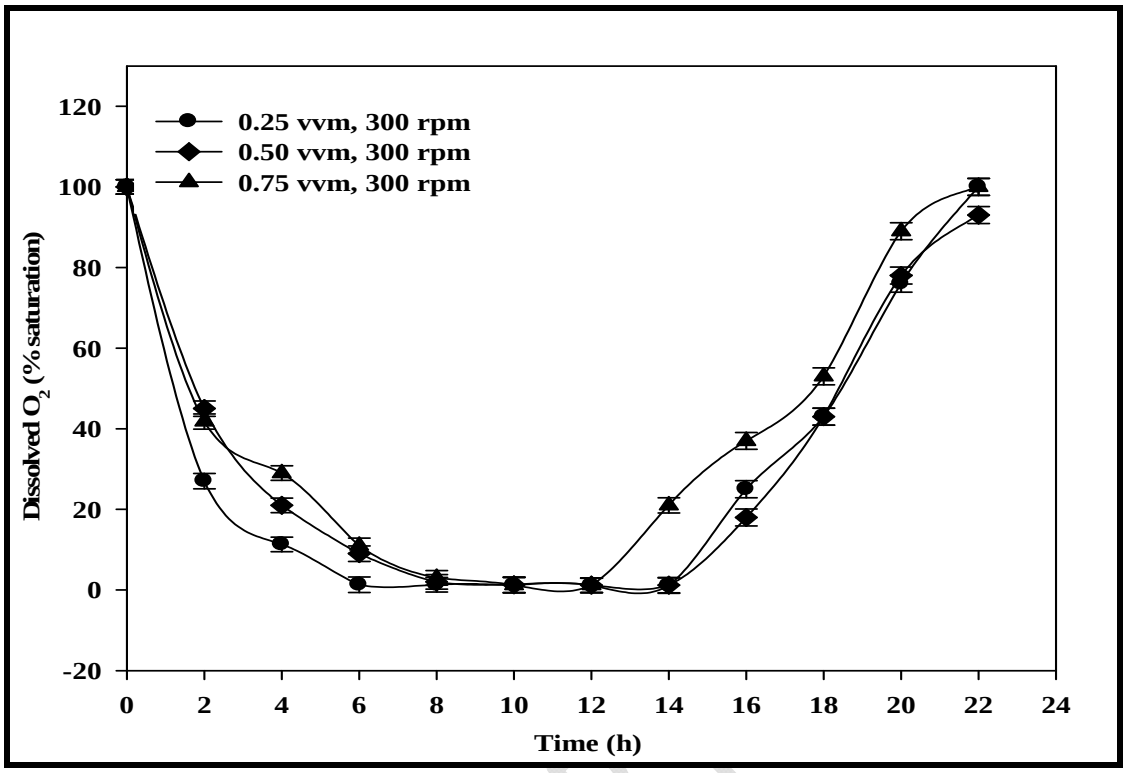
0.25 vvm, 300 rpm
0.50 vvm, 300 rpm
0.75 vvm, 300 rpm
Dissolved O2 (% saturation)
Time (h)

Fig. 6 (C)

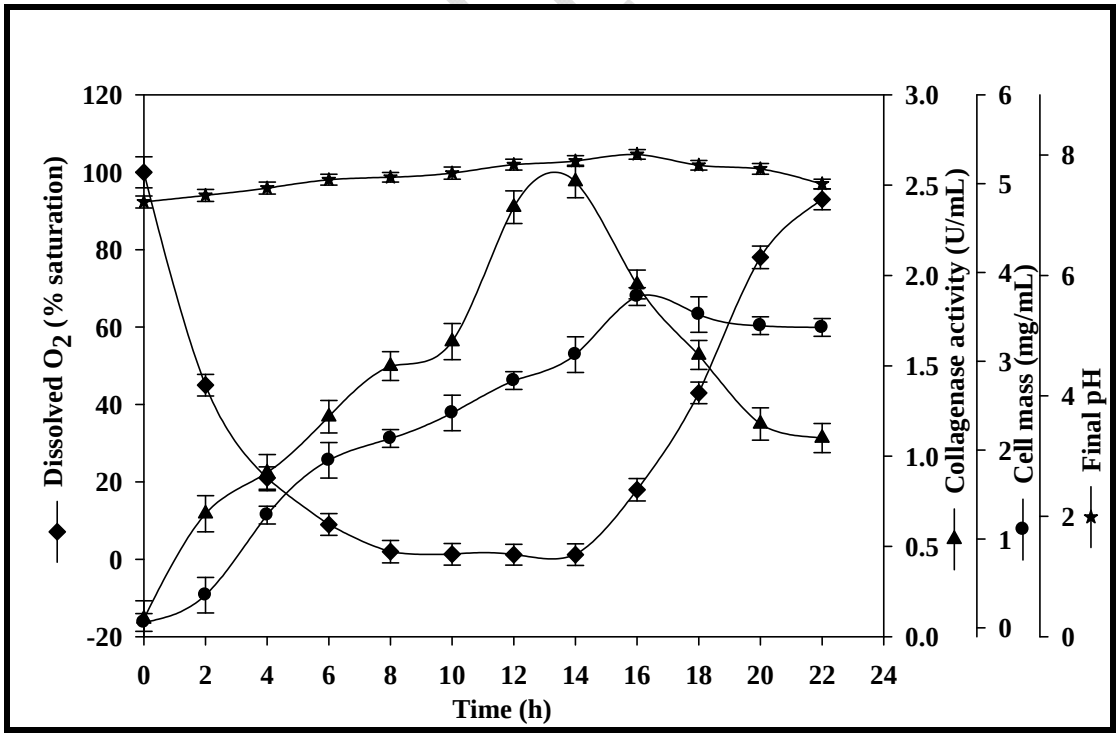
Dissolved O2 (% saturation)
Collagenase activity (U/mL)
Cell mass (mg/mL)
Final pH
Time (h)

Fig. 7