Effect of hydromechanical forces on the production of filamentous haemagglutinin and pertussis toxin of *Bordetella pertussis*

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SUMMARY

The production of *Bordetella pertussis* extracytoplasmic filamentous haemagglutinin (FHA) and pertussis toxin (PT) in a bioreactor under stirring conditions was studied in order to investigate the effect of hydromechanical forces on yields of both antigens. It was shown that FHA loses its haemagglutinin activity when the power transmitted by the agitator and the aerator per unit volume increases, whereas PT production is not affected. The loss of FHA activity can be explained by the action of shear forces on the filamentous structure of this antigen.

NOMENCLATURE

C^*	dissolved oxygen saturation concentration
C_l	dissolved oxygen concentration
D	impeller diameter
$\bar{E}$	power transmitted by the agitator and the aerator per unit of liquid volume
E_m	maximum local energy dissipation rate per unit of liquid volume
K_La	volumetric oxygen transfer coefficient
N	impeller speed
Pg	power input in aerated system
qO_2m	maximum specific oxygen consumption rate
R_e	Reynold number ($D^2N \, \rho/\mu$)
VVM	volume of air per volume of fermentation broth per minute
Xm	maximum of biomass concentration
λo	Kolmogorov-microscale
μ	fermentation broth viscosity
ν	fermentation broth kinematic viscosity
ρ	fermentation broth density
expt	experiment

INTRODUCTION

Bordetella pertussis is the main etiologic agent of whooping cough in children. This organism produces two distinct haemagglutinins, with different chemical and biological

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properties. One of them, the filamentous haemagglutinin (FHA), appears as filaments 2 nm in diameter and 100 nm in length by electron microscopy [3]. The other haemagglutinin, pertussis toxin (PT), exhibits a spherical structure, 6 nm in diameter, and has a relatively low haemagglutinin activity (HA). These two haemagglutinins are secreted into the surrounding medium during cell growth. They are considered the major determinants of pathogenesis as well as efficient immunogens [9,14,17]. For this reason, both antigens were included as components of an acellular pertussis vaccine [16].

Although these proteins can be produced with static or agitated cultures, either their relative or actual amounts obtained depend strongly on the culture conditions. Only static cultures employing large numbers of Roux bottles have been successful in producing sufficient amounts of FHA [1,2].

When large scale growth of virulent *B. pertussis* is required for antigen production, it is very important to keep environmental conditions homogeneous. This is attained by adequate agitation to ensure both appropriate mixing of contents and suitable heat and mass transfer rates. However, under these conditions FHA loses both its HA and its serological reactivity [2,5]. Although some improvement in medium composition has partially contributed to solve this problem [5,6], the cause of this loss of HA remains unknown.

FHA is a filamentous protein. This structure could render it sensitive to shearing. For this reason, the loss of HA in relation to the shear forces generated in aerated-stirred fermenters was examined in this work, in the belief that these studies would contribute to improve bioreactor design in order to optimize the process of antigen production.

Since the use of the recommended Stainer Scholte (SS) medium [18] for cell and antigen production gave low yields

[7], studies regarding supplementation of this medium with yeast extract have also been included.

MATERIALS AND METHODS

Bacterial strain and culture procedures

B. pertussis strain 8132 (Pasteur Institute Collection) was maintained in 15% glycerol, 1% casamino acids at -70 °C. Cells were grown for 72 h and subcultured for 30 h at 36 °C on Bordet-Gengou agar (Difco laboratories, Detroit, Michigan) supplemented with 10 g L^{-1} peptone and 15% (v/v) defibrinated sheep blood. Cells harvested from these plates were transferred to 250-ml Erlenmeyer flasks containing 125 ml of Stainer Scholte [18] medium modified to contain 0.00, 0.50, 0.75, 1.00 and 1.25 g L^{-1} yeast extract (Difco). They were incubated for 24 h with a rotary shaker at 160 rpm. 25 ml of these cultures were used to seed 1-L Erlenmeyer flasks containing 250 ml of each medium.

Batch fermenter cultures of B. pertussis were carried out in a 6-L bioreactor (LKB 1601 ULTROFERM, LKB, Bromma, Sweden), having a working volume of 2.5 L. The growth was followed for 24 h until the stationary phase was reached. Dissolved oxygen was measured using a galvanic electrode. In all the experiments the volumetric oxygen transfer coefficient (K_La) was kept constant at 60 h^{-1} by varying either the agitator speed or the air flow rate. Foam was controlled by automatic addition of antifoam (1520 Dow Corning, Siliar). Temperature was maintained at 36 °C. The fermenter was inoculated with bacteria from a 24-h shake flask containing SS medium modified by the addition of 0.75 g L^{-1} yeast extract. In all experiments (shake flask or fermenter), the initial optical density (at 650 nm) was approximately 0.1–0.2. Cellular growth was followed by measuring the optical density at 650 nm (OD_{650}) with a Pye Unicam SP6-250 visible spectrophotometer (1 unit OD_{650} = 3.0×10^9 organism ml^{-1}).

Haemagglutination

The haemagglutinin activity of the culture supernatant samples was determined by microtiter assay using goose erythrocyte [15].

Pertussis toxin assay

PT antigen in culture supernatant was detected by ELISA using fetuin as a solid phase. The wells of flat-bottomed Immulon-Immunoplates were coated with 100 μl of fetuin (25 μl L^{-1} in 0.05 M carbonate buffer, pH 9.6). The plates were kept overnight at 4 °C in a humidified box, then the wells were washed six times with PBS containing 0.05% (v/v) Tween-20 (PBST). Test samples were serially diluted in PBST, 100 μl being then added to wells. Plates were incubated at 37 °C for 1 h in a humidified box and washed as described above. 100 μl goat anti-BP antibody was added to each well, plates were incubated at 37 °C for 1 h, and washed. Anti-goat IgG-peroxidase conjugate in PBST (100 μl) was added to each well; plates were then incubated at 37 °C, and washed. The enzyme reaction was initiated by the addition of 10 μl of O-phenylene diamine (0.1 mg ml^{-1})

and 100 μl of H_2O_2 (30 vol in 100 ml citrate–phosphate buffer, pH 5). After 15 min in the dark at room temperature, the reaction was stopped by the addition of 50 μl of H_2SO_4 (4 N). PT contents were determined measuring the absorbance at 492 nm, employing PT (Sigma) as reference.

Immunoblotting

SDS-PAGE was performed according to Laemmli [10] using 10% polyacrylamide slab gels. Transfer of proteins from SDS-polyacrylamide gels to nitrocellulose sheets was performed as described by Towbin et al. [19]. Immobilized proteins were incubated with mouse monospecific antibodies. Bound antibodies were detected using alkaline phosphatase-linked goat anti-mouse IgG (ProtoBlot System, Promega, Madison). The antibodies used were anti-FHA kindly provided by Nicole Guisó (Pasteur Institute, Paris, France).

Volumetric oxygen transfer coefficient (K_La)

K_La was determined by using the sulfite oxidation technique according to Cooper et al. [4]. Measurements were performed at 37 °C, with a working volume of 2.5 L.

Respirometry

Oxygen uptake was determined by using a Gilson Differential, Single Valve Style Respirometer (IGR-20 respirometer). The oxygen consumption rate was estimated according to Umbreit et al. [20]. In order to calculate the maximum specific oxygen consumption rate (qO_2m), the assay was carried out with samples corresponding to the logarithmic growth phase. The biomass concentration was determined by dry weight.

RESULTS AND DISCUSSION

Effect of yeast extract addition to SS medium

Fig. 1 shows that yeast extract addition to the SS medium increases both the production of cells and the extracytoplasmic levels of PT and HA. This effect was shown to be dependent on yeast extract concentration. When organisms were grown in SS medium containing 0.75 g L^{-1} of yeast extract, both cell and antigen yields were maximal. A reduction of the lag period was also observed (data not shown).

These results indicate that the SS medium lacks some factor(s) which is (are) present in the yeast extract, an observation which warrants further research. Because of the above-mentioned results, the present study was carried out with the modified culture medium.

Oxygen requirements of B. pertussis

In order to avoid O_2 limitation during growth the necessary K_La to be used at the fermenter scale was estimated as follows:

$$K_La = \frac{qO_2m\ Xm}{C^* - C_1}$$

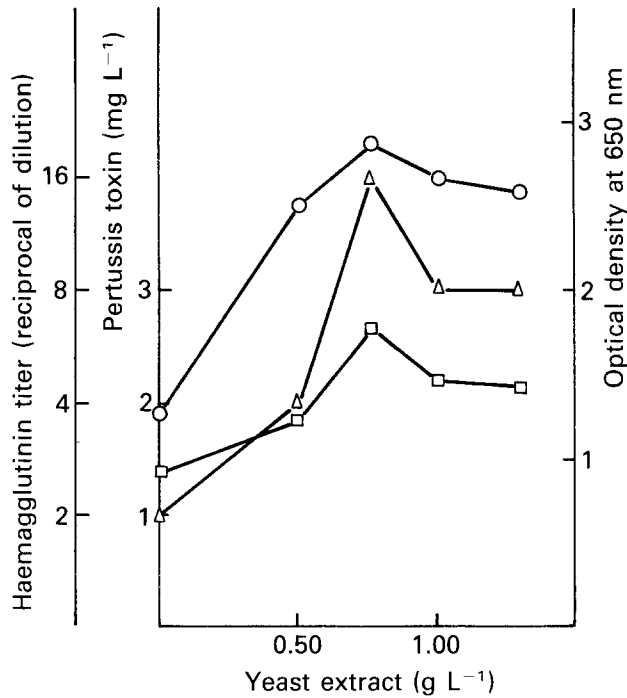The qO_2m determined by respirometric assay was 3.82 mmol

Fig. 1. Effect of yeast extract concentration on maxima values of biomass concentration (O), HA titer (△) and PT level (□). All the results presented are the mean of four experiments, carried out in 1000-ml shaken flask, containing 250 ml of culture medium.

h^{-1} g. The dissolved oxygen saturation concentration (C^*) at 36 °C was estimated to be 0.182 mmol L^{-1}. Taking into account that the maximum biomass concentration (Xm) reached in the culture medium was 2 g L^{-1}, and that the recommended minimum value of the dissolved oxygen tension (C_l) for *B. pertussis* was 30% of C^*, it was found that K_La value should be kept equal to or above 60 h^{-1}.

Effect of operational conditions on cell and antigen production in stirred fermenter

Experiments were carried out in batch stirred aerated reactors at different aeration flow rates and agitator speeds,

as mentioned in Materials and Methods. Table 1 shows the effect of the different operational conditions on the maximum values of cell concentration, HA and PT levels. Values mentioned were reached at the same time during the course of the experiments (Fig. 2).

As can be seen, the maximum concentrations of biomass and extracytoplasmic PT obtained were similar for the four operational conditions tested. However, the maximum extracellular HA varied according to the different culture conditions. This variation can only be accounted for by the extracellular FHA level, since the other haemagglutinin present (PT) did not change with the different operational condition. Furthermore, its haemagglutinin activity is six- or seven-fold lower than the FHA activity. Gorringe et al. [5] suggested that the loss of haemagglutinin activity in shaken flask cultures could be due to aggregated FHA without activity, which would appear on the surface of the liquid as a result of its hydrophobicity. Accordingly, the loss of HA would be related to the air–liquid interface area produced in shaken cultures. That is to say, HA would decrease as the air–liquid interface area increases. However, under the experimental conditions employed in this study, this relationship does not hold for the results obtained (Table 1). Thus, at the same K_La, for higher agitation speed and lower air flow rate, the air–liquid interface would be expected to show a small decrease or no change. Therefore, according to Gorringe's conclusion HA should not be lower.

Since the results obtained showed a loss of HA at increasing values of agitator speed, surface aggregation of FHA could not explain the loss of activity observed in stirred bioreactors. It is known that preparations of FHA which lacked the 220 and 210 kDa bands show reduced specific haemagglutinin [3]. Since no proteolytic activity could be detected at any instance, the fact that other phenomena are responsible for the HA decrease may be inferred.

As FHA is a filamentous protein, shear forces generated in the system studied could cause chain scission and consequently the observed fall in HA. According to the Kolmogorov's model [11] in turbulent flow, shear effects on

TABLE 1

Effect of operating conditions on the production of biomass and extracytoplasmic PT and FHA of *B. pertussis* in a stirred bioreactor

expt	N* rpm	VVM* L L^{-1} min^{-1}	R_e^{**} 10^{-4}	Pg w	OD max	HA max titer	PT max mg L^{-1}
1	210	1.00	1.7	0.88	2.40	32	2.05
2	270	0.80	2.2	18.90	2.40	8	2.09
3	320	0.48	2.6	33.00	2.42	4	2.05
4	450	0.30	3.7	96.00	2.46	1	2.02

All the results are means of four experiments.
* K_La was kept constant at 60 h^{-1}.
** At every tested condition, fermentation broth density was 1 Kg m^{-3} and viscosity remained constant at 1.3 10^{-3} Ns m^{-2}. Fermentation broth exhibits a Newtonian flow behavior.
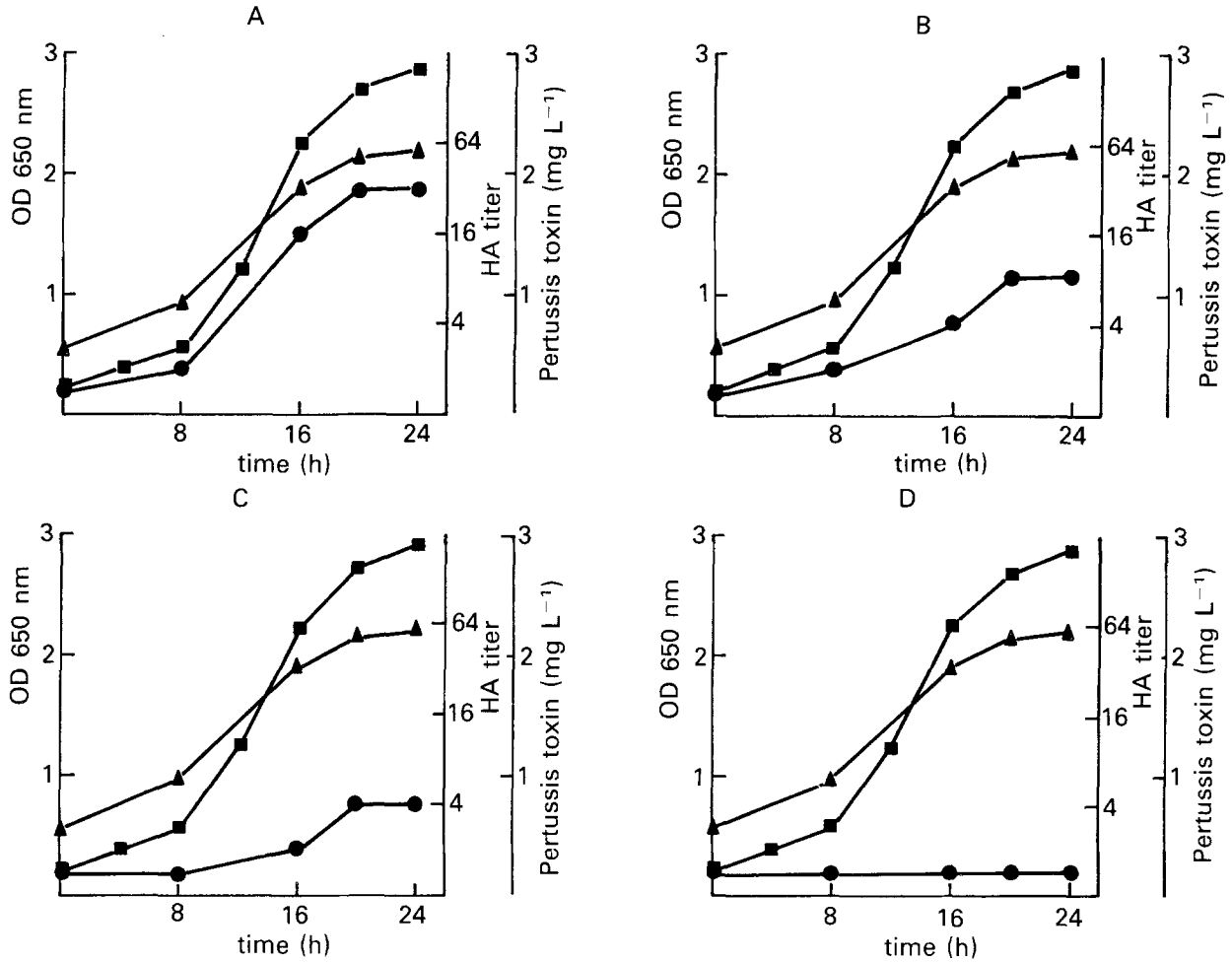
Fig. 2. Effect of the operating condition on time course of growth (■), HA titer (●) and PT production (▲) of *B. pertussis* in a 6-L stirred fermenter, with a working volume of 2.5 L. A) N = 210, VVM = 1 L L⁻¹ min⁻¹; B) N = 270, VVM = 0.8 L L⁻¹ min⁻¹; C) N = 320, VVM = 0.48 L L⁻¹ min⁻¹; D) N = 450, VVM = 0.3 L L⁻¹ min⁻¹. The results are the mean of four experiments.

suspended solids of an order of magnitude of the smallest eddies are to be expected. An order-of-magnitude calculation of Kolmogorov-microscale (λo) was performed for each cultural condition by:

$$\lambda o = \left[\frac{\nu^3 \rho}{\bar{E}}\right]^{0.25}$$

Since the mixing regime is turbulent ($R_e > 10^4$) (Table 1), and λo was of the same order of magnitude as FHA, under every operation condition, protein damage is assumed as it enters a high turbulence area, where it is caught into eddies moving in different directions. The disruption would occur as a result of highly localized velocity gradients within or between eddies. The kinetic energy would then cause chain scission with a consequent loss of HA.

Maximum local energy dissipation rate (E_m) is generally considered to be the best correlating parameter for shear stress in the turbulent regime. An estimation of E_m has been given by Liepe [12]:

$$E_m = 0.5\,\bar{E}\,(D/T)^{-3}$$

D and T are geometric factors which remained constant under the given conditions, so that the correlating parameter considered was merely the power transmitted by the agitator and the aerator per unit of liquid volume ($\bar{E}$).

Fig. 3 shows the variation of PT and FHA maxima related to this parameter. As can be seen, no differences were observed in PT concentration at increasing $\bar{E}$, whereas HA titer decreased sharply. Consequently, the FHA level appears to be highly dependent on the shear forces generated in the culture broth.

In order to confirm these results a control experiment was carried out. Samples taken from cultures of *B. pertussis* grown under stationary conditions were centrifuged at 7000 g for 15 min at 4 °C. Supernatants free of organisms were obtained according to microscopic observations. These supernatants showed high HA titer (initial time of incubation, Table 2). Samples of supernatant (2.5 L) were incubated in a stirred fermenter for 6 h under the same operating
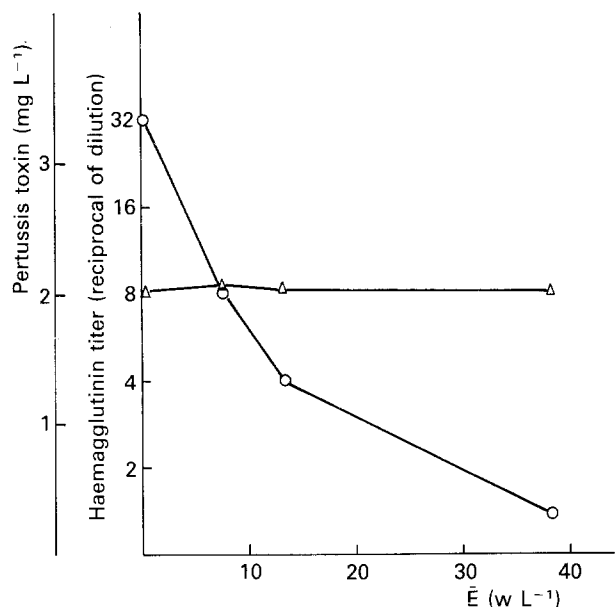
Fig. 3. Effect of the power input per unit of liquid volume on the maxima PT concentration ($\triangle$), and HA titer (○), in a 6-L stirred fermenter, with a working volume of 2.5 L. The results are the mean of four experiments.

TABLE 2

Effect of operating conditions on HA titer of supernatant from *Bordetella pertussis*

expt*	N rpm	VVM $L\ L^{-1}\ min^{-1}$	HA titer time of incubation (h)	
			0	6
control	0	0.00	32	32
1	210	1.00	32	16
2	270	0.80	32	8
3	320	0.42	32	4
4	450	0.30	32	1

The results are expressed as the mean HA titer from two experiments. * Expt 1, 2, 3 and 4 were carried out in a 6-L fermenter, with a working volume of 2.5 L. All the experiments were carried out at 36 °C.

conditions employed previously in this study. As a control, one sample was maintained static for 6 h at 36 °C. HA titer was evaluated at the start and at the end (6 h) of incubation in all cases. Table 2 shows that at the static condition no decrease in HA titer occurs during incubation. However, supernatants incubated with agitation showed a decrease in HA titer. In accordance with the results obtained previously, the HA titer showed a more rapid decline at higher stirrer speeds.

To gain further insight in connection to these results, analysis by SDS-PAGE and immunoblotting were carried out using samples taken at three different conditions: from the start of the experiment, 6 h under stationary incubation

and 6 h under the highest stirring condition. The SDS-PAGE pattern of the sample from the highest stirring condition revealed a partial disappearance of the components of molecular weight ranging from 220 to about 180 kDa in relation to the sample taken at the start and after 6 h of static incubation. These two latter samples revealed no differences by SDS-PAGE analysis nor by immunoblotting. Fig. 4 shows that the sample under agitating conditions did not contain bands corresponding to higher molecular weight components of FHA. This loss was accompanied by an increase of FHA components of molecular weight lower than 97 kDa.

From these results we can infer that the loss of haemagglutinin activity in a stirred bioreactor would be caused by mechanical damage to the FHA protein structure. In connection with serological activity of FHA, these results are important considering that only the higher-molecular-weight polypeptides of FHA seem to be capable of eliciting protective antibodies [9,13]. Knowledge about the extracytoplasmic FHA breakdown by shear forces would make it easier to select operational conditions to which this antigen can be exposed. Moreover, the introduction of microscale turbulence as a criterion of detrimental limit that leads to FHA damage is an important conceptual step by itself. This is of particular relevance for designing and scaling-up the fermentation process. In this respect a low shear bioreactor
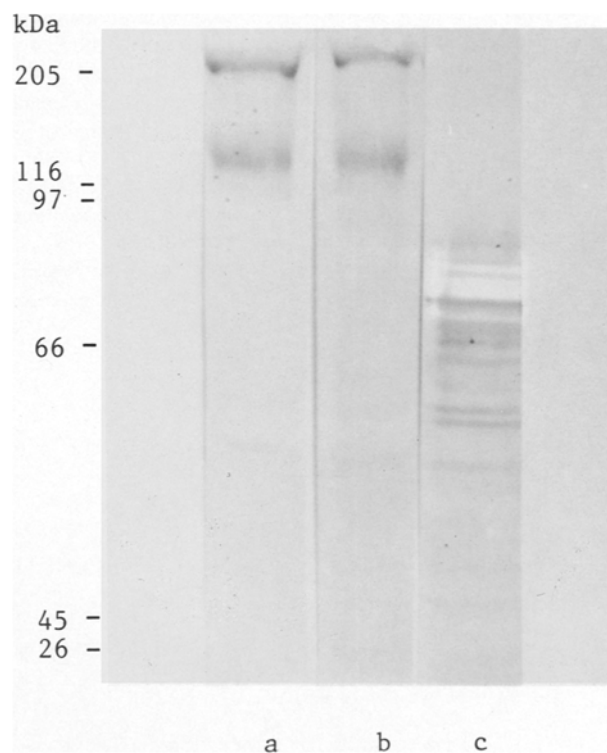

Fig. 4. Immunoblot analysis of static culture supernatant of *B. pertussis* after being treated as follows: lane a) supernatant without any treatment; lane b) supernatant incubated without agitation for 6 h at 36 °C; lane c) supernatant incubated for 6 h at 36 °C under the following condition: $N = 450$, VVM $= 0.3\ L\ L^{-1}\ min^{-1}$.

could be an interesting alternative for production of both antigens.

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