

Effects of pH on Biomass, Maximum Specific Growth Rate and Extracellular Enzyme Production by Three Species of Cutaneous Propionibacteria Grown in Continuous Culture

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Three cutaneous propionibacteria, *Propionibacterium acnes*, *Propionibacterium avidum* and *Propionibacterium granulosum*, were grown in chemostats using semi-synthetic medium at various pH values. Growth occurred between pH 4.5 and 7.5 for *P. acnes* and pH 5.0 and 8.0 for *P. avidum* and *P. granulosum*. The highest μ_{\max} was at pH 6.0 for the three species. Maximum biomass production was obtained at pH 6.0 for *P. acnes* and *P. avidum* and at pH 7.5 for *P. granulosum*. Extracellular enzyme production occurred over the entire pH growth range when denaturation of the enzymes was taken into account. However, detectable activity of the enzymes was found in a narrower range of pH due to the denaturation of the enzymes at low or high pH values. The highest production of enzymes occurred at pH values between 5.0 and 6.0, apart from the production of hyaluronate lyase of *P. granulosum* (pH 6.0 to 7.0) and the proteinase of *P. acnes* and *P. avidum* (pH 5.0 to 7.5). *Propionibacterium acnes* produced a lipase, hyaluronate lyase, phosphatase and proteinase activity. *Propionibacterium avidum* produced a lipase and proteinase activity. *Propionibacterium granulosum* produced a lipase and hyaluronate lyase.

INTRODUCTION

Propionibacterium acnes, *Propionibacterium avidum* and *Propionibacterium granulosum* are inhabitants of normal sebaceous follicles of human skin (McGinley *et al.*, 1978). *Propionibacterium acnes* and *P. granulosum* are found in acne lesions (Marples *et al.*, 1973; Leyden *et al.*, 1975; Gloor & Franke, 1978). Holland *et al.* (1978) hypothesized that microenvironmental changes in sebaceous follicles are major factors in determining the physiology of the bacterial inhabitants with consequent effects on the follicle. pH would be an important variable in this environment.

Cutaneous propionibacteria produce extracellular enzymes including lipase (Hassing, 1971; Kellum *et al.*, 1970; Ingham *et al.*, 1981; Greenman *et al.*, 1981), hyaluronidase (hyaluronate lyase) (Puhvel & Reisner, 1972; Ingham *et al.*, 1979; Greenman *et al.*, 1981), proteinase, DNAase (Marples & McGinley, 1974) and acid phosphatase (Ingham *et al.*, 1980; Greenman *et al.*, 1981), which may play a role in the initiation of inflammation in acne (Holland *et al.*, 1981).

The aim of this comparative investigation was to determine the effect of changing the pH on the physiology of *P. acnes*, *P. avidum* and *P. granulosum* grown in chemostat cultures and of particular interest was extracellular enzyme production.

METHODS

Organisms and media. *Propionibacterium acnes* (laboratory strain P37), *P. avidum* [laboratory strain PF77(i)] and *P. granulosum* [laboratory strain PF208(ii)] were isolated, identified and maintained as described by Greenman *et al.* (1981).

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Table 1. Dilution rates used in wash-out experiments to determine the maximum specific growth rates of *P. acnes*, *P. avidum* and *P. granulosum*

pH	Maximum specific growth rate, μ_{\max} (h^{-1}) of:		
	<i>P. acnes</i>	<i>P. avidum</i>	<i>P. granulosum</i>
4.5	0.23	—	—
5.0	0.29	0.28	0.24
5.5	0.25	0.28	0.21
6.0	0.26	0.28	0.23
6.5	0.23	0.23	0.23
7.0	0.24	0.24	0.24
7.5	0.28	0.29	0.29
8.0	—	0.27	0.18

Table 2. Denaturation constants (λ) for the extracellular enzymes of *P. acnes*, *P. avidum* and *P. granulosum* at different pH values

The denaturation constant was zero for proteinase of *P. acnes* and *P. avidum* at all pH values.

pH	Denaturation constant, λ (h^{-1})*					
	<i>P. acnes</i>			<i>P. avidum</i>	<i>P. granulosum</i>	
	Lipase	Hyaluronate lyase	Phosphatase	Lipase	Lipase	Hyaluronate lyase
4.5	0.51	0.244	0.015	0.116	0.056	0.06
5.0	0.35	0.14	0.004	0.09	0.057	0.04
5.5	0.09	0.005	0.064	0.03	0.03	0.003
6.0	0.33	0.034	0.16	0.116	0.02	0.005
6.5	0.64	0.315	0.42	0.48	0.02	0.038
7.0	1.56	0.420	0.46	0.56	0.105	0.64
7.5	3.52	1.4	0.81	0.94	0.15	0.73

* For methods of calculating λ , see Eaves *et al.* (1979).

The medium was that used by Eaves *et al.* (1979). In addition, sterile glucose solution (10%, w/v) was added aseptically to a final concentration of 0.2% (w/v) for *P. granulosum*, which requires glucose for good growth (Greenman *et al.*, 1981).

Continuous culture apparatus and conditions. Organisms were grown in a one litre culture vessel with control modules for temperature, pH, gas flow and stirrer rate (Series 500, L.H. Engineering, Stoke Poges, Buckinghamshire). The growth conditions were those used by Eaves *et al.* (1979). The pH was preselected and maintained at a constant level (± 0.1 unit) over the range pH 4.0 to 8.5 in 0.5 unit increments by the automatic addition of 2 M-NaOH or 2 M-HCl. Dilution rates were set to allow a specific growth rate of $0.33 \mu_{\max}$ for each particular pH condition used.

Determination of biomass and maximum specific growth rates. These were determined as described by Greenman *et al.* (1981). The dilution rates used for washout are given in Table 1. Production rate of biomass was expressed as g dry wt cells $\text{l}^{-1} \text{h}^{-1}$.

Extracellular enzymes. Lipase (EC 3.1.1.3), hyaluronate lyase (EC 4.2.2.1) and acid phosphatase (EC 3.1.3.2) activities were assayed by the methods used by Holland *et al.* (1979). Proteinase activity was assayed by the method of Millet (1970). All activities were expressed as $\mu\text{mol end-product h}^{-1} (\text{mg dry wt cells})^{-1}$ apart from the proteinase activity, which was expressed as units $\text{h}^{-1} (\text{mg dry wt cells})^{-1}$, where a unit is defined as $(A_{440} \times 100)/(T \times 0.5)$ and T is the time of incubation of the assay mixture. Production rate (h^{-1}) of this enzyme was expressed as units $\text{h}^{-1} (\text{mg dry wt cells})^{-1}$.

Production rate of the other extracellular enzyme activities was expressed as $\mu\text{mol end-product h}^{-1} (\text{mg dry wt cells})^{-1} \text{h}^{-1}$. The extracellular enzymes had different stabilities at different pH values. The corrected production rates were calculated from the measured activities using a correction formula $(\lambda + D)$ measured activity/ D , where D is the dilution rate and λ is the denaturation constant for the enzyme at a given pH (Eaves *et al.*, 1979). The denaturation constants are given in Table 2. Proteinase was stable under the conditions tested and no correction had to be made.

Steady state. Biomass and extracellular enzyme activities were determined on samples taken from the chemostat during the particular conditions of pH that were selected. Only after a minimum of six culture volume changes had occurred between particular conditions was a steady state condition considered to be achieved.

Statistical analysis. Linear regression analysis was used to determine the slope of the curve for the wash-out data for calculation of μ_{\max} .

For biomass and extracellular enzyme production data the standard error of the mean was computed and, with the number of samples the $\pm 95\%$ confidence limits were calculated.

RESULTS

Cell biomass and extracellular enzyme production were measured for each species of *Propionibacterium* grown at a steady state, with the pH of the growth medium increased from pH 4.0 to 8.5 in 0.5 unit increments. All steady states were at $0.33 \mu_{\max}$ for the particular pH and organism studied. This enabled comparison of the performance of the three species at different pH values (Evans, 1976; Herbert, 1976; Tempest, 1976).

Maximum specific growth rates

The results for μ_{\max} are shown in Fig. 1. *Propionibacterium avidum* and *P. granulosum* grew within the same pH range (pH 5.0 to 8.0), whilst *P. acnes* grew within the range pH 4.5 to 7.5. The curves show a broad optimum pH range for the three species with the highest values of μ_{\max} (0.23 h^{-1} for *P. acnes*; 0.21 h^{-1} for *P. avidum*; 0.15 h^{-1} for *P. granulosum*) recorded at pH 6.0.

Biomass production

Propionibacterium acnes and *P. avidum* behave similarly with optimum production of biomass at pH 6.0 (Fig. 2). In contrast, *P. granulosum* shows maximum biomass production at pH 7.0 to 7.5. Biomass production increases steadily up to pH 7.0 and after 7.5 rapidly decreases to zero.

Extracellular enzyme production

The production of extracellular enzyme was determined from enzyme activity in the culture supernatant and the dilution rate used. Detailed results for *P. acnes* are shown in Figs 3 to 5 together with the corrected production rate of extracellular enzymes taking into account their denaturation. Results for *P. avidum* and *P. granulosum* are summarized in the text.

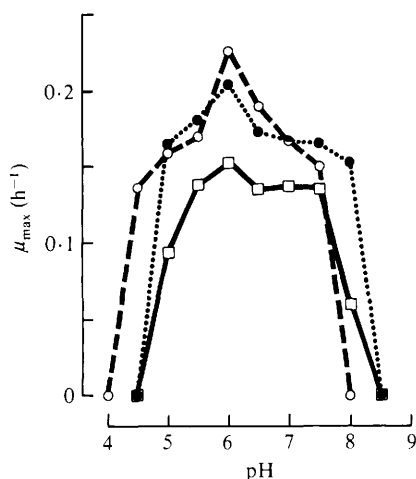


Fig. 1

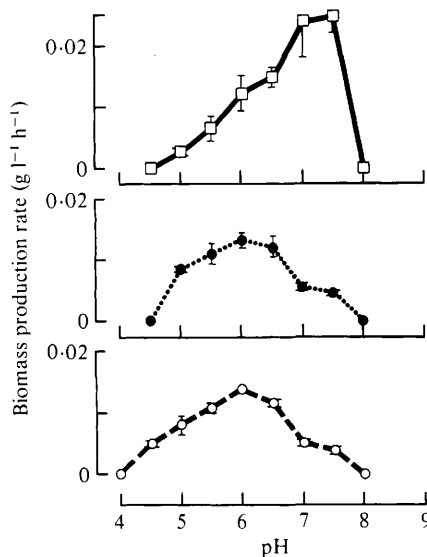


Fig. 2

Fig. 1. Effect of pH on the maximum specific growth rate of *P. acnes* (○), *P. avidum* (●) and *P. granulosum* (□).

Fig. 2. Effect of pH on biomass production at $0.33 \mu_{\max}$ of *P. acnes* (○), *P. avidum* (●) and *P. granulosum* (□). The bars represent 95% confidence limits about the mean.

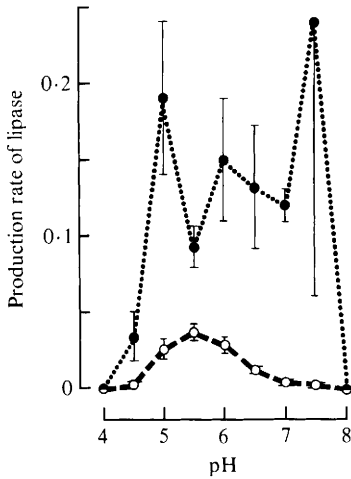


Fig. 3

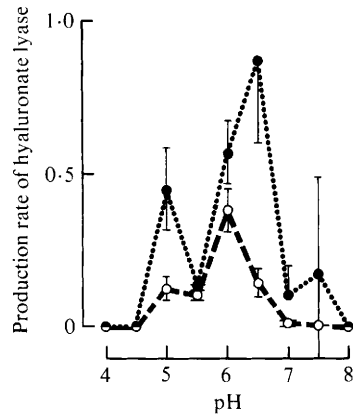


Fig. 4

Fig. 3. Effect of pH on the production rate of lipase at $0.33 \mu_{\max}$ by *P. acnes*, uncorrected (○) and corrected (●) for enzyme denaturation. Lipase production rate is expressed as μmol oleic acid released h^{-1} (mg dry wt cells) $^{-1}$ h^{-1} . The bars represent 95% confidence limits about the mean.

Fig. 4. Effect of pH on the production rate of hyaluronate lyase at $0.33 \mu_{\max}$ by *P. acnes*, uncorrected (○) and corrected (●) for enzyme denaturation. Hyaluronate lyase production rate is expressed as μmol *N*-acetylglucosamine released h^{-1} (mg dry wt cells) $^{-1}$ h^{-1} . The bars represent 95% confidence limits about the mean.

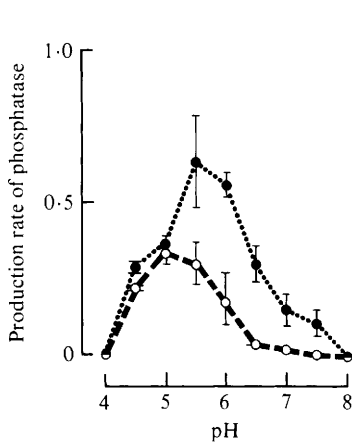


Fig. 5

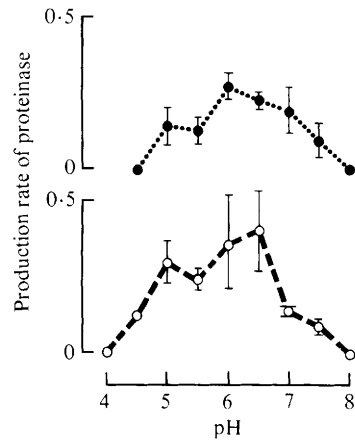


Fig. 6

Fig. 5. Effect of pH on the production rate of phosphatase at $0.33 \mu_{\max}$ by *P. acnes*, uncorrected (○) and corrected (●) for enzyme denaturation. Phosphatase rate is expressed as μmol *p*-nitrophenol released h^{-1} (mg dry wt cells) $^{-1}$ h^{-1} . The bars represent 95% confidence limits about the mean.

Fig. 6. Effect of pH on the production rate of proteinase activity at $0.33 \mu_{\max}$ by *P. acnes* (○) and *P. avidum* (●). Production rate of proteinase activity is expressed as units h^{-1} (mg dry wt cells) $^{-1}$ h^{-1} . The bars represent 95% confidence limits about the mean.

The highest production rate of lipase by the three species of bacteria was at pH 5.5 with production occurring over a narrow range pH 5.0 to 6.5 for *P. acnes* (Fig. 3). Similar results were obtained for *P. avidum* and *P. granulorum* (data not shown). However, after allowance for enzyme denaturation, the corrected production rate varied little for *P. acnes* between culture pH values 5.0 to 7.5 (Fig. 3). Maximum corrected production rates occurred at pH 6.5 for *P. avidum*

and pH 5.5 for *P. granulosum* with a range of pH 5.0 to 7.5. Over the range tested, *P. granulosum* was capable of most lipase production, three times that of *P. acnes*.

Propionibacterium acnes and *P. granulosum* produced extracellular hyaluronate lyase. The highest production of this enzyme was at pH 6.0 for *P. acnes* (Fig. 4) and pH 6.0 to 7.0 for *P. granulosum*. Production occurred at pH values from 5.0 to 6.5 for *P. acnes* and 5.0 to 7.5 for *P. granulosum*. The corrected production rate optimum was pH 7.0 for *P. granulosum* and two optima were obtained for *P. acnes*, pH 5.0 and 6.5. The range of pH for corrected production rate was 5.0 to 7.5 for both species. Production of hyaluronate lyase was greater by *P. acnes* at all pH values apart from pH 7.0, when the corrected production rate by *P. granulosum* exceeded that of *P. acnes*.

Extracellular phosphatase was detected only in *P. acnes* cultures (Fig. 5). The highest production was at pH 5.0 to 5.5 and the range of production was pH 4.5 to 6.0; very little enzyme was found in cultures maintained above pH 6.5. The optimum for corrected production rate was pH 5.5 and the range was pH 4.5 to 7.5.

Extracellular proteinase activity was shown in *P. acnes* and *P. avidum* cultures (Fig. 6). In both species, this activity was stable and the production occurred between pH 4.5 to 7.5 for *P. acnes* and pH 5.0 to 7.5 for *P. avidum*. The highest production rate for *P. acnes* was over a pH range of 5.0 to 6.5 and, for *P. avidum*, 6.0 to 7.0.

Apart from the hyaluronate lyase of *P. acnes*, all the enzymes were produced over the entire pH growth range of the bacteria. *Propionibacterium acnes* grew between pH 4.5 to 7.5, but the hyaluronate lyase was not detected at pH 4.5.

DISCUSSION

Apart from the μ_{\max} determination, all experiments were performed at $0.33 \mu_{\max}$ for the particular pH maintained in the culture. This enabled comparison of the three species. However, strict comparison cannot be made between *P. granulosum* and the other two species because it was essential to include 0.2% (w/v) glucose in the medium for *P. granulosum* (Greenman *et al.*, 1981).

All statements concerning the ranges and optimum pH values for a particular species must be viewed in the light of the experiments carried out at 0.5 pH unit increments. *Propionibacterium acnes* grew at pH 4.5 and not at pH 4.0. However, it is not possible to calculate at which pH between 4.5 and 4.0 growth is stopped.

It should be noted that μ_{\max} values were obtained for *P. avidum* and *P. granulosum* at pH 8.0 by the wash-out method (Table 1) and yet at $0.33 \mu_{\max}$, values of zero biomass production are given at pH 8.0 (Fig. 2). At $0.33 \mu_{\max}$, the cultures of *P. avidum* and *P. granulosum* gradually decreased in biomass over an extended period of time (48 to 60 h) and it was concluded that the cultures were not in steady states. This inability of the cultures to maintain biomass at $0.33 \mu_{\max}$ cannot be explained adequately. It might be explained by minor oscillations in the pH of the culture near the limiting pH for growth. It should be noted that the pH control is accurate at ± 0.1 pH unit and the experiment spans a much greater time than the wash-out study for μ_{\max} when there is less chance of pH fluctuation in a short time.

Previous investigations have shown that the three species produce different combinations of extracellular enzymes (Holland *et al.*, 1979; Greenman *et al.*, 1981) and that *P. granulosum* requires a sugar carbon source in a tryptone-based medium, whilst the other two species do not (Holland *et al.*, 1979; Greenman *et al.*, 1981). Another difference of *P. granulosum* compared with *P. acnes* and *P. avidum* has been demonstrated in this study. Maximum biomass production in the experimental medium occurs at pH 6.0 with *P. acnes* and *P. avidum*, with low production at pH 7.0 and 7.5. In contrast, *P. granulosum* biomass production is highest at pH 7.0 and 7.5. Differences also occur with extracellular enzyme production.

For all the extracellular enzymes studied, the pH affects the production rate. In addition, apart from the proteinase activities of *P. acnes* and *P. avidum*, the enzymic activity is negatively modulated by the effect of pH on the stability of the enzyme. This reduces the quantity of active enzyme available and the pH range at which activity appears. For *P. acnes* the

production of active enzyme is limited to below pH 6.5 with maximum active enzyme production at pH 5.0 for phosphatase, pH 5.5 for lipase and pH 6.0 for hyaluronate lyase. *Propionibacterium avidum* lipase production is similar to *P. acnes* and about six times greater. *Propionibacterium granulosum* extracellular lipase and hyaluronate lyase production differs from *P. acnes* enzyme production is that it occurs above pH 6.5 and maximum production of active hyaluronate lyase is greatest at pH 7.0.

The proteinase activity produced by *P. acnes* and *P. avidum* is stable over the pH range tested and, of all the enzymes studied, is produced over the widest pH range. This might be explained by more than one proteinase being produced over different pH ranges.

It is tempting to extrapolate from these laboratory acquired results to predictions and explanations of the microbial ecology of human skin. There are inherent difficulties in such extrapolation; not the least is that only one environmental variable has been examined namely, pH. However, the results suggest that pH could be one of the main factors in maintaining *P. acnes* as the most common species of *Propionibacterium* on the sebaceous follicle-rich areas of human skin. McGinley *et al.* (1978) showed that, of the three species, *P. acnes* had the highest frequency of isolation and population density on these skin sites, and the skin pH is acidic (Noble, 1968) – values ranging from pH 4.0 to 6.8 (mean 5.26) in adults were reported by Holland & Cunliffe (1982) and means ranging from pH 4.6 to 5.1 by Abe *et al.* (1980). The ability of *P. acnes* to grow at a lower pH than *P. granulosum*, its relatively high μ_{max} at the lower pH limit for growth, its extracellular enzyme production and stability, which is greatest at lower pH values, all suggest that *P. acnes* is well adapted to the pH found on human skin.

McGinley *et al.* (1978) reported that *P. granulosum* was less frequently isolated from sebaceous follicle-rich areas of skin and Gloor & Franke (1978) and Leyden *et al.* (1975) showed that *P. granulosum* had a higher frequency of isolation from acne lesions compared with normal skin. It is possible that acne-inflamed lesions have a pH of or approaching 7.4, because of the disruption of the follicular wall and cellular infiltrate. This might give *P. granulosum* an advantage in colonizing acne lesions, because its biomass and extracellular enzyme production is greatest at pH 7.0 to 7.5. *Propionibacterium avidum* is limited to most moist areas of skin (McGinley *et al.*, 1978) and it would be unwise to evoke arguments based on environmental pH to explain its limited habit.

Marples *et al.* (1971) showed that *P. acnes* was the major organism on human skin responsible for production of free fatty acid in skin surface lipids. The lipase of this organism hydrolyses sebum triglycerides produced by the sebaceous glands. The free fatty acid content of skin surface lipids varies from person to person [1.1 to 32.6% (w/w) (Cotterill *et al.*, 1971)]. This variation could be explained not only by the number of bacteria on the skin but also the active lipase production rate which can be affected by the environmental pH. It would be predicted that a high free fatty acid content in skin surface lipids would be produced from follicles at pH values around 5.5. The bacterial analysis of single follicles is possible (Puhvel *et al.*, 1975). If follicular pH values could be obtained then the prediction could be tested.

Some of the extracellular enzymes of the cutaneous propionibacteria may have properties in addition to their enzymic function. These may include the ability to activate the alternate complement sequence, to be antigenic, or to be chemo-attractant to phagocytic cells. These properties may play an important role in causing inflammation in acne (Holland *et al.*, 1981). The different stabilities of these enzymes at different pH values may only be with respect to their enzymic (catalytic) function; the molecules may still retain other biological properties. The presence of 'denatured' enzyme becomes apparent after applying the correction formula to the measured rates of enzyme production. In every case, the corrected production rate is significantly higher (up to sixfold) than the measured rate.

This relatively high amount of inactive enzyme may be of importance in the pathogenesis of acne if its production occurs *in vivo* and if it does possess other biological properties.

Until the effects of the other enzymes on the follicular environment are known there can be no speculation about the effects that pH will have on these enzymes *in vivo*.

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