Lait (1998) 78, 217-226

Original article

Optimized standard conditions for determination of nitrate reduction in propionibacteria

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(Received 18 February 1997; accepted 11 November 1997)

Abstract — Nitrate reduction is currently considered to be a key characteristic for the determinative grouping of strains of the *Propionibacterium* but many controversies exist regarding nitrate reduction by members of this genus. The aim was thus to determine the influence of different media, nitrate concentrations and pH values under aerobic and anoxic conditions, using the conventional technique and the diazotization coupling method, on the nitrate reducing capability of propionibacteria. The low concentrations of nitrite formed during nitrate reduction by the propionibacteria could not be accurately determined using the conventional technique. This method was found to be reliable only when the initial KNO_3 concentration exceeded 1.0 mmol/L. In contrast, with high concentrations of nitrite, the diazotization coupling method was found to be too sensitive. Nitrate reduction in the yeast extract lactate (YEL) medium with added glucose and KNO_3, was not effective as only low concentrations of nitrite were produced. Good nitrate reduction was obtained on both the YEL and yeast extract (YE) media with or without the addition of KNO_3 (pH 8.0) to the test media. The presence of nitrite in the YEL culture media of the five *Propionibacterium* type strains, under aerobic conditions (pH 8.0) with 20 mmol/L KNO_3, was determined using the diazotization coupling method. The type strains of *P. acidipropionici*, *P. acnes* and *P. freudenreichii* subsp. *freudenreichii* were able to reduce nitrate. In contrast, the *P. jensenii* and *P. thoenii* type strains were not able to reduce nitrate under these conditions. Furthermore, *P. acidipropionici* and *P. acnes* were both able to further reduce the formed nitrite to gaseous nitrogen. It was found that nitrate reduction was strongly influenced by environmental factors such as oxygen, nitrate concentration, pH, media composition, incubation period and the presence of glucose. Data are presented which permit a more standardized determination of nitrate reduction of propionibacteria. © Inra/Elsevier, Paris.

***Propionibacterium* / nitrate reduction / environmental factors**

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Résumé — Optimisation des conditions standard pour la détermination de la réduction du nitrate par les bactéries propioniques. La réduction du nitrate est généralement considérée comme étant une caractéristique clef pour déterminer des groupes de *Propionibacterium* mais de nombreuses controverses existent concernant la réduction du nitrate par les membres de ce genre de bactéries. Le but était donc de déterminer l'influence de différents milieux, de la concentration en nitrate et des valeurs de pH dans des conditions aérobies et anoxiques sur la capacité de réduction du nitrate par les bactéries propioniques, et ce, en utilisant la technique conventionnelle et la méthode de couplage de diazotisation. Les faibles concentrations en nitrite formé au cours de la réduction du nitrate par les bactéries propioniques ne pouvaient pas être déterminées avec précision par la technique conventionnelle. Cette méthode s'est avérée fiable uniquement lorsque la concentration initiale de KNO_3 excédait 1,0 mmol/L. En revanche, avec une concentration élevée de nitrite, la méthode de couplage de diazotisation s'est avérée trop sensible. La réduction du nitrate dans le milieu YEL additionné de glucose et de KNO_3 n'était pas efficace puisque seulement de faibles concentrations de nitrite étaient produites. Une bonne réduction de nitrate était obtenue tant sur le milieu YEL que sur celui à l'extrait de levure avec ou sans addition de KNO_3 (pH 8,0). La présence de nitrite dans le milieu de culture YEL des cinq souches types de *Propionibacterium*, en aérobiose (pH 8,0) avec 20 mmol/L de KNO_3, a été déterminée en utilisant la méthode de couplage de diazotisation. Les souches types de *P. acidipropionici*, de *P. acnes* et de *P. freudenreichii* subsp. *freudenreichii* étaient capables de réduire le nitrate dans ces conditions, contrairement aux souches types de *P. jensenii* et de *P. thoenii*. De plus, *P. acidipropionici* et *P. acnes* étaient capables de réduire le nitrite formé en azote gazeux. La réduction du nitrate s'est révélée fortement influencée par des facteurs environnementaux tels que l'oxygène, la concentration en nitrate, le pH, la composition des milieux, la durée d'incubation et la présence de glucose. Les données présentées permettent une meilleure détermination standardisée de la réduction du nitrate par les bactéries propioniques. © Inra/Elsevier, Paris.

***Propionibacterium* / réduction du nitrate / facteurs environnementaux**

1. INTRODUCTION

The ability of an organism to reduce nitrate is influenced by various environmental factors, including: medium composition [2]; pH [3]; dissolved oxygen concentration [9, 25]; light intensity [25]; incubation period [2]; nitrate concentration [1]; and other factors [7, 10, 18, 26].

Many controversies exist [16, 23, 27] regarding nitrate reduction by members of the *Propionibacterium*. This is an extremely important phenotypic characteristic as it is used as one of the major differential characteristics to separate the four dairy species [6]. Kaspar [16], however, reported that all the dairy *Propionibacterium* species reduced nitrate to nitrite and further to nitrous oxide (N_2O). In contrast, Van Gent-Ruijters et al. [27] reported that only one of three strains of '*P. pentosaceum*' was able to reduce nitrate to nitrite and further to N_2O or N_2. In the current classification system, '*P. arabinosum*' and '*P. pentosaceum*' strains were consolidated [19] to form the *P. acidipropionici* species, although nitrate reduction was negative for the '*P. arabinosum*' strains, while certain of the '*P. pentosaceum*' strains were able to produce nitrite as well as nitrogen [4]. According to Cummins and Johnson [6], the other dairy species could not reduce nitrate, with the exception of *P. freudenreichii* subsp. *freudenreichii*.

As nitrate reduction is used as one of the major characteristics to separate the species of the *Propionibacterium* [6], the influence of environmental parameters on nitrate reduction needs to be examined. These conditions should thus be optimized so as to validate the application of nitrate reduction as a differential charac-

teristic. The aim of this study was to investigate the influence of oxygen, nitrate concentration, pH, media composition, incubation period, as well as the addition of glucose, on the ability of *Propionibacterium* strains to reduce nitrate.

2. MATERIALS AND METHODS

2.1. Bacterial strains, culture maintenance and growth media

Five *Propionibacterium* type strains (table I), *Escherichia coli* ATCC 11775 and *Leuconostoc dextranicum* DSM 20484 were included in this study. The *E. coli* and the *L. dextranicum* strains were included as positive [9, 17] and negative controls, respectively.

Lyophilized cultures [14] were used to inoculate yeast extract lactate medium (YEL) to obtain working cultures. The YEL medium (g/L) consisted of: yeast extract 6.0; sodium lactate (70% v/v) 20.0; peptone 2.0; KH_2PO_4 10.0; and Tween 80 1.0 mL. The medium was prepared anaerobically [13] and the pH adjusted to 7.2 before sterilization. The dairy propionibacteria were incubated for 4 days at 30 °C and the *P. acnes* strain incubated for 6 days at 37 °C in an anaerobic cabinet, using 10% H_2: 10% CO_2: 80% N_2 as gas phase. *Escherichia coli* was incubated aerobically for 48 h at 37 °C on nutrient agar (Merck) with 1% (m/v) glucose (pH 7.0) and *L. dextranicum* grown on tomato juice broth (Difco) medium (pH 4.8) and incubated aerobically at 25 °C for 7 days.

Culture purity was regularly checked and the production of propionic and acetic acids as metabolites were monitored gas chromatographically for each propionibacterial strain [5].

2.2. Influence of external parameters

The influence of external parameters on nitrate reduction by the *P. acidipropionici* type strain was determined using a modified De Man-Rogosa-Sharpe medium (M-MRS) [8]. The M-MRS-medium consisted of 20 mmol/L sodium phosphate buffer with (g/L): MRS (Merck) 25.0 and added sodium lactate (70% v/v) 20.0. The M-MRS was then used as basis for the addition of different concentrations of potassium nitrate (KNO_3) (0.0, 1.0, 5.0, 10.0 and 15.0 mmol/L). The initial pH value of each assay was also varied (pH 5.0, 6.0, 7.0, 8.0 and 9.0).

A pre-inoculum was prepared by growing each culture under anoxic conditions for five days on M-MRS plates. A 10% standard inoculum was prepared and the M-MRS-medium inoculated, followed by incubation under aerobic as well as anoxic conditions, at 30 °C. All tests were done in duplicate. Unless otherwise stated, a 10% standard inoculum ($OD_{640} = 0.21$) of all strains grown for 5 days was used throughout this study.

Nitrate reduction was determined by measuring for the presence of nitrite, using the

Table I. *Propionibacterium* type strains used in this study.

Tableau I. Souches types de *Propionibacterium* utilisées dans cette étude.

Culture number	Species	Source
56	*P. acnes*	ATCC 6919
80	*P. jensenii*	DSM 20535
419	*P. thoenii*	NCFB 568
423	*P. freudenreichii* subsp. *freudenreichii*	ATCC 6207
424	*P. acidipropionici*	ATCC 25562

ATCC, American Type and Culture Collection, Rockville, MD, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; NCFB, National Collection of Food Bacteria, Reading, UK.

conventional technique as described by Gerhardt et al. [12]. The intensity of the red color was scored visually on a scale from 0 to 5. Nitrate reduction was also assayed using the diazotization coupling method described by Nicholas and Nason [20], using a Beckman DU8 spectrophotometer.

Nitrate reduction was also determined using nitrate broth (Difco) as growth medium. This was performed at different pH values (6.0, 7.0, 8.0 and 9.0) and varying potassium nitrate concentrations (10 and 20 mmol/L). The pH was compensated before autoclaving. The influence of various glucose concentrations (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0%) (m/v) in nitrate broth on nitrate reduction by *P. acidipropionici* was also evaluated. All tests were performed under aerobic, as well as anoxic conditions. Anoxic conditions were obtained by incubation of the samples in an anaerobic cabinet. Aerobic conditions were obtained by aerobic incubation of the static culture.

2.3. Influence of media composition

The optimal pH and KNO_3 concentration, as determined in the previous section, was applied in this section. The influence of seven different media combinations on the nitrate reducing capability of *P. acidipropionici* during growth phase studies was evaluated in this section. The different media included: YEL broth with the addition of both 10% glucose and 20 mmol/L KNO_3; YEL broth with or without 20 mmol/L KNO_3; yeast extract (6.0 g/L) and glucose (10 g/L) (YEG) with or without 20 mmol/L KNO_3 and yeast extract (6.0 g/L) (YE) with or without 20 mmol/L KNO_3. In specific cases KNO_3 was added to the test media to determine if the presence of nitrate in the growth media influenced or enhanced nitrate reduction.

A 20% standard inoculum (OD 1.0 at 640 nm) was prepared and the various media combinations were incubated at 30 °C. Samples were withdrawn at four hourly intervals for the analysis of nitrite. All tests were performed in triplicate and uninoculated tubes served as controls. In this section, the presence of nitrite was determined using the diazotization coupling method [20].

3. RESULTS AND DISCUSSION

3.1. Influence of growth medium and nitrite assay techniques

The data obtained when using nitrate broth (Difco) under aerobic and anoxic conditions at different pH values and nitrate concentrations, showed that when using both the conventional (data not shown) and the diazotization coupling method, the *L. dextranicum* strain did not reduce nitrate while the *E. coli* strain strongly reduced the nitrate. This confirmed the use of these strains as negative and positive controls [11, 21].

During the study, it was found that the conventional method was unsuitable for nitrite determination from propionibacteria when using the M-MRS-medium, as no nitrate reduction was observed for the *P. acidipropionici* type strain at any pH value or at the different KNO_3 concentrations. In contrast, nitrite was formed in low concentrations by *P. acidipropionici* in the nitrate broth (*table II*) under both aerobic and anoxic conditions at pH 7.0 and 8.0 and only under aerobic conditions at pH 9.0. No nitrite was detected at pH 6.0. The data thus indicate that the type of medium used must be taken into consideration as it has an influence on the ability of an organism to reduce nitrate. Blaszczyk [2] also reported similar results. Nitrate reduction by the *P. acidipropionici* strain, using the nitrate broth under the same conditions, was also determined using the diazotisation coupling method (*table III*). Similar results were obtained but this method was more sensitive, showing the highest nitrate reduction at pH 8.0 with 20 mmol/L KNO_3 under aerobic conditions. In contrast, when this method was used to determine nitrate reduction for the *E. coli* strain, it was found that the results were not repeatable even when the samples were diluted. Thus, based on the results, it was concluded that the conventional technique might not be sensitive

Table II. Nitrate reduction by *P. acidipropionici* in nitrate broth after incubation at different pH and KNO_3 values (mmol/L) under aerobic and anoxic conditions using the conventional nitrite determination technique (data visually scored from 0 to 5).

Tableau II. La réduction du nitrate par *P. acidipropionici* dans le bouillon Nitrate après incubation à différentes valeurs de pH et de KNO_3 (mmol/L) sous des conditions aérobies et anoxiques utilisant la technique conventionnelle de détermination du nitrite (données catégorisées visuellement de 0 à 5).

pH	KNO_3 concentration (mmol/L)			
	Aerobic		Anoxic	
	10	20	10	20
6.0	0	0	0	0
7.0	1	1	1	1
8.0	1	1	1	1
9.0	1	1	0	0

Table III. Effect of aerobic and anoxic conditions, different KNO_3 concentrations (mmol/L) and pH values on the ability of *P. acidipropionici* to reduce nitrate in nitrate broth, using the diazotization coupling method (values are in nmol nitrite per mL and are the average of duplicate tests).

Tableau III. Effets des conditions aérobies et anoxiques, des différentes concentrations de KNO_3 (mmol/L) et des valeurs de pH sur la capacité du *P. acidipropionici* à réduire le nitrate du bouillon Nitrate en utilisant la méthode de couplage de déazotisation (les valeurs sont en nmol de nitrite par mL et représentent la moyenne d'un test répété deux fois).

pH	KNO_3 concentration			
	Aerobic		Anoxic	
	10 mmol/L ± SD	20 mmol/L ± SD	10 mmol/L ± SD	20 mmol/L ± SD
6.0	0.00	0.00	0.00	0.00
7.0	7.08 ± 0.71	9.09 ± 2.84	10.09 ± 2.13	15.1 ± 4.96
8.0	12.85 ± 1.06	18.85 ± 1.06	1.31 ± 0.35	4.57 ± 3.55
9.0	4.57 ± 0.71	3.57 ± 0.71	0.00	0.00

enough when low concentrations of nitrite are formed during nitrate reduction by propionibacteria. The data showed that when the conventional method was used, it was only reliable when the initial KNO_3 concentration in the medium exceeded 1.0 mmol/L. In contrast, however, when high concentrations of nitrite were formed, as found for the *E. coli* strain, the diazotization coupling method may again be too sensitive, even if the samples were diluted. Based on these results, it is recommended that the conventional method be used to determine nitrate reduction when testing *E. coli* and that the diazotization coupling method be used when studying nitrate reduction by *Propionibacterium* strains.

3.2. Influence of environmental factors

The YEG medium (with and without added nitrate) was not very effective for nitrite determination (*figure 1*), as only low concentrations were produced when the *P. acidipropionici* culture was grown in this medium. Nitrate reduction was obtained in both the YEL and YE media (both without glucose addition) with or without KNO_3 (pH 8.0) (*figure 1*). No nitrite was detected when the *P. acidipropionici* culture was grown in the YEL medium with 10% glucose and 20 mmol/L KNO_3. This was also found when varying glucose concentrations were used in the nitrate broth. Care must thus be taken when glucose is added to a medium as no or very little nitrate reduction would be found.

The best medium for the assay of nitrate reduction, by the *P. acidipropionici* type strain, was found to be the YEL medium (*figure 1*) with the addition of 20 mmol/L KNO_3 (pH 8.0) to the medium without glucose under aerobic conditions. This medium was thus used during further experimental studies. Under these conditions, enough biomass for the extraction and purification of the enzyme was also produced. In the case of the YE medium, nitrate reduction was found but very little biomass was produced.

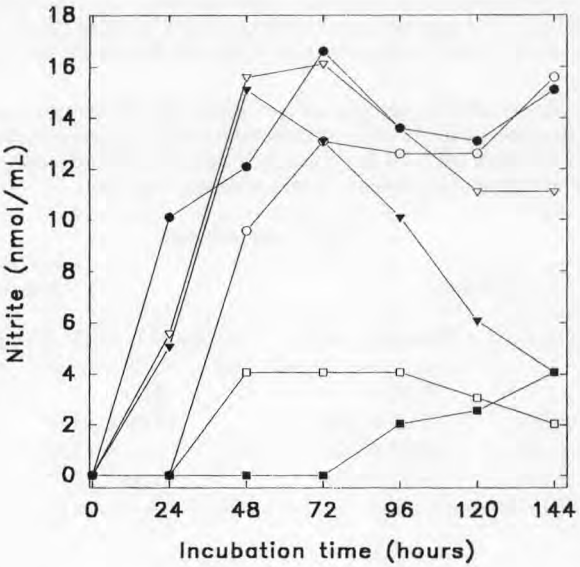

Figure 1. The influence of media composition on the ability of *Propionibacterium acidipropionici* to reduce nitrate (● YEL plus 20 mmol/L KNO_3; ○ YEL minus 20 mmol/L KNO_3; ■ YEG plus 20 mmol/L KNO_3; □ YEG minus 20 mmol/L KNO_3; ▼ YE plus 20 mmol/L KNO_3; ∇ YE minus 20 mmol/L KNO_3). Values represent the average of duplicate tests (SD < 3%).

Figure 1. L'influence de la composition des milieux sur la capacité de *Propionibacterium acidipropionici* à réduire le nitrate (● YEL plus 20 mmol/L de KNO_3; ○ YEL moins 20 mmol/L de KNO_3 ; ■ YEG plus 20 mmol/L de KNO_3 ; □ YEG moins 20 mmol/L de KNO_3 ; ▼ YE plus 20 mmol/L de KNO_3 ; ∇ YE moins 20 mmol/L de KNO_3). Les valeurs représentent la moyenne d'un test répété deux fois (écart type < 3%).

The five *Propionibacterium* type strains (*table I*) were inoculated into YEL medium with 20 mmol/L KNO_3 under aerobic conditions (pH 8.0) and the presence of nitrite was determined according to the diazotisation coupling method at regular intervals (*figure 2*). The type strains of *P. acidipropionici, P. acnes* and

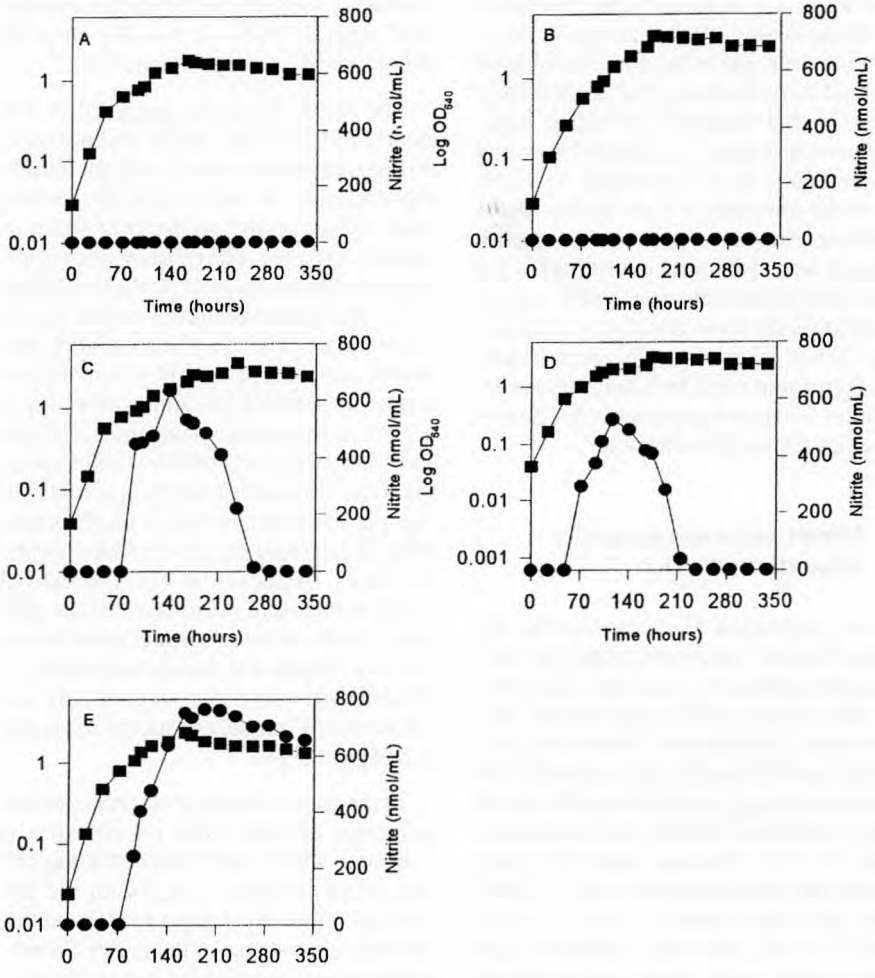

Figure 2. Nitrate reduction and growth by the *Propionibacterium* type strains in YEL medium with 20 mmol/L KNO_3 at 30 °C and pH 8.0 (A = *P. thoenii* NCFB 568; B = *P. jensenii* DSM 20535; C = *P. acidipropionici* ATCC 25562; D = *P. acnes* ATCC 6919; E = *P. freudenreichii* subsp. *freudenreichii* ATCC 6207) [■ = log OD_{640}; ● = nitrite - nmol/mL]. Values represent the average of triplicate tests (SD < 5%).

Figure 2. Réduction du nitrate et croissance des souches types de *Propionibacterium* dans le milieu YEL, avec 20 mmol/L de KNO_3 à 30 degrés C et à un pH de 8.0 (A = *P. thoenii* NCFB 568 ; B = *P. jensenii* DSM 20535 ; C = *P. acidipropionici* ATCC 25562 ; D = *P. acnes* ATCC 6919 ; E = *P. freudenreichii* subsp *freudenreichii* ATCC 6207) [■ = Log DO_{640} ; ● = Nitrite - nmol/mL]. Les valeurs représentent la moyenne d'un test répété à trois reprises (écart type < 5%).

P. freudenreichii subsp. *freudenreichii* were all able to reduce nitrate (*figure 2C, D, E*). In contrast, *P. thoenii* and *P. jensenii* (*figure 2A, B*) were not able to reduce nitrate under these conditions. Furthermore, *P. acidipropionici* and *P. acnes* (*figure 2C, D*) were both able to reduce the accumulated nitrite, as can be seen from the decrease in the concentration of nitrite. The reduction of the accumulated nitrite by *P. freudenreichii* subsp. *freudenreichii* progressed at a much lower rate (*figure 2E*). This can probably be ascribed to the presence of a less active nitrite reductase enzyme system in this specific strain. It was also found in this part of the study that nitrate reduction could only be detected for the three positive type strains after 70 h of incubation. This is an important factor that must be taken into consideration when using nitrate reduction as a species differential character.

3.3. Nitrate reduction as species separation criterion

The separation of species within the genus *Propionibacterium*, using the identification system of Cummins and Johnson [6], is based on five phenotypic characteristics, including the ability to reduce nitrate. Several reports can be found in the literature relating to problems on the use of nitrate reduction as differential characteristic [22, 23]. The data from this study confirm the identification system of Cummins and Johnson [6] in that *P. thoenii* and *P. jensenii*, under the conditions used in this study, are the only type strains not able to reduce nitrate. The variation of an organism's ability to reduce nitrate can present a serious problem if this phenotypic characteristic is used to differentiate between species. This is especially true for the genus *Propionibacterium*, as is found with the species *P. acidipropionici*, *P. jensenii* and *P. thoenii*. If a *Propionibacterium* strain is not able to reduce nitrate, that strain will be classified as a member of the *P. jensenii* or *P. thoenii* species, even though the majority of the characters would be similar to that of *P. acidipropionici* [5]. This incorrect identification based on one phenotypic characteristic, will only be apparent if a numerical analysis based on a wider range of phenotypic data are performed [5].

The results from this study (*table III*) clearly indicate that media composition, aerobic and anoxic conditions, the nitrate concentration, as well as pH, have a dramatic effect on the ability of a strain to reduce nitrate. This study therefore confirms the findings of Riedel and Britz [24], that nitrate reduction in the genus *Propionibacterium* can be a variable phenotypic characteristic with external parameters influencing the ability of an organism to reduce nitrate. The results of Focht and Verstraete [10] and Blösl and Conrad [3] were also confirmed in this study, in that pH has a drastic effect on the capability of microorganisms to reduce nitrate. Similarly, Allison and Macfarlane [1] found that culture pH influenced the products of dissimilatory nitrate reduction in *P. acnes*. Nitrate was converted to nitrite at alkaline pH (pH 7.5), whereas nitrous oxide was the product of nitrate reduction at a pH of 6.0 [1].

In the genus *Propionibacterium*, nitrate reductase appears to be constitutive as indicated in the results obtained in the presence and absence of nitrate in the test medium. Similarly, Kaspar [16] stated that the nitrate reductase of the genus *Propionibacterium* seems to be either constitutive or derepressed by anaerobiosis. According to Kaspar [16], nitrate appeared to stimulate synthesis of the nitrate reductase in *P. acidipropionici*, while oxygen inhibited nitrous oxide production in both *P. acidipropionici* and *P. thoenii*. This is in contrast to the results of Kaneko and Ishimoto [15], who found that the presence of nitrate in the growth medium

itself had no effect on the nitrate reductase activity of *P. acidipropionici*. The nutritional composition of the culture medium was also found to have an effect on the ability of an organism to reduce nitrate, as found for '*P. pentosaceum*' [27] and *Paracoccus denitrificans* [2].

Blaszczyk [2] also showed that incubation period is of great importance. Cultures of *Paracoccus denitrificans* grown in nutrient broth required only a 12-h incubation period, whereas cultures grown in mineral media, supplemented with ethanol, sodium acetate and methanol, required longer periods to reduce the same amount of nitrate. In this study, *P. acnes*, *P. acidipropionici* and *P. freudenreichii* subsp. *freudenreichii* were only able to reduce measurable amounts of nitrate after 70 of incubation (*figure 2C, D, E*). In contrast, it was also reported that specific growth rates of *P. acnes* were higher in the presence of nitrate [1] than in medium without nitrate. Thus, the incubation period is also of critical importance when using nitrate reduction as a differential phenotypic characteristic, especially if the incubation period is too short.

The suppression of nitrate reduction by glucose was also observed during this study, confirming the results of Schulp and Stouthamer [26]. Van Gent-Ruijters et al. [27] found that complex media were unsuitable for use in nitrate reduction studies in '*P. pentosaceum*' and that nitrate reductase was partially repressed by glucose [26].

It is thus recommended that environmental factors, such as the pH, nitrate concentration, aerobic or anaerobic conditions and the growth medium, should be standardized when using nitrate reduction as criterion for differentiation between species in the genus *Propionibacterium*. Using the optimum conditions, as found for the type strains in this study, more strains of the genus *Propionibacterium* should be evaluated for the ability to reduce nitrate. It is also recommended that the nitrate reductase gene (NAR) of *P. acidipropionici* be further studied and compared with that of other genera and species.

ACKNOWLEDGMENT

This research was funded by the South African Foundation for Research Development.

REFERENCES

[1] Allison C., Macfarlane G.T., Dissimilatory nitrate reduction by *Propionibacterium acnes*, Appl. Environ. Microbiol. 55 (1989) 2899–2903.

[2] Blaszczyk M., Effect of medium composition on the denitrification of nitrate by *Paracoccus denitrificans*, Appl. Environ. Microbiol. 59 (1993) 3951–3953.

[3] Blösl M., Conrad R., Influence of an increased pH on the composition of the nitrate-reducing microbial populations in an anaerobically incubated acidic forest soil, Syst. Appl. Microbiol. 15 (1992) 624–627.

[4] Breed R.S., Murray E.G.D., Smith N.R., Genus 1. *Propionibacterium* Orla-Jensen 1909, in: Breed R.S., Murray E.G.D., Smith N.R., (Eds.) Bergey's Manual of Determinative Bacteriology, 7th edn. Williams & Wilkins Co, Baltimore, MD, USA, 1957.

[5] Britz T.J., Riedel K.H.J., *Propionibacterium* species diversity in Leerdammer cheese, Int. J. Food Microbiol. 22 (1994) 257–267.

[6] Cummins C.S., Johnson J.L., Genus 1. *Propionibacterium* Orla-Jensen 1909, in: Sneath P.H.A., Mair N.S., Sharpe M.E., Holt J.G., (Eds.) Bergey's Manual of Systematic Bacteriology, Vol. 2, Williams & Wilkins Co, Baltimore, MD, USA, 1986.

[7] Dalsgaard T., Bak F., Nitrate reduction in a sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, isolated from rice paddy soil: sulfide inhibition, kinetics, and regulation, Appl. Environ. Microbiol. 60 (1994) 291–297.

[8] De Man J.D., Rogosa M., Sharpe M.E., A medium for the cultivation of *Lactobacilli*, J. Appl. Bacteriol. 23 (1960) 130–135.

[9] Denis K.S., Dias F.M., Rowe J.J., Oxygen regulation of nitrate transport by diversion of electron flow in *Escherichia coli*, J. Biol. Chem. 265 (1990) 18095–18097.

[10] Focht D.D., Verstraete W., Biochemical ecology of nitrification and denitrification, Adv. Microbial. Ecol. 1 (1977) 135–214.

[11] Garvie E.I., Genus *Leuconostoc* Van Tieghem 1878, in: Sneath P.H.A., Mair N.S., Sharpe M.E., Holt J.G. (Eds.) Bergey's Manual of Systematic Bacteriology, Vol. 2, Williams & Wilkins Co, Baltimore, MD, USA, 1986.

[12] Gerhardt P., Murray R.G.E., Costilow R.N., Nester E.W., Wood W.A., Krieg N.R., Phillips G.B., Manual of Methods for General Bacteriology, American Society for Microbiology, Washington, DC, USA, 1981.

[13] Holdeman L.V., Cato E.P., Moore W.E.C., Anaerobe Laboratory Manual, 2nd edn., Virginia Polytechnic Institute and State University, Blacksburg, Virginia, 1977.

[14] Joubert W.A., Britz T.J., A simple and inexpensive method for the long term preservation of microbial cultures, J. Microbiol. Methods 7 (1987) 73–76.

[15] Kaneko M., Ishimoto M., A study on nitrate reductase from *Propionibacterium* acidi-propionici, J. Biochem. 83 (1978) 191–200.

[16] Kaspar H.F., Nitrite reduction to nitrous oxide by propionibacteria: detoxification mechanism, Arch. Microbiol. 133 (1982) 126–130.

[17] Kirstein K., Bock E., Close genetic relationship between *Nitrobacter hamburgensis* nitrite oxidoreductase and *Escherichia coli* nitrate reductases, Arch. Microbiol. 160 (1993) 447–453.

[18] McCarty G.W., Bremner J.M., Effects of Mn^{2+} and Mg^{2+} on assimilation of KNO_3 and NH_4^+ by soil microorganisms, Proc. Natl. Acad. Sci. USA 90 (1993) 9403–9407.

[19] Moore W.E.C., Holdeman L.V., *Propionibacterium*, in: Buchanan R.E., Gibbons N.E. (Eds.) Bergey's Manual of Determinative Bacteriology, 8th edn., Williams & Wilkins Co, Baltimore, MD, USA, 1974.

[20] Nicholas D.J.D., Nason A., Determination of nitrate and nitrite, in: Colowick S.P., Kaplan N.O. (Eds.), Methods in Enzymology, Vol. III 144, Academic Press, New York, 1957.

[21] Orskov F., Genus 1. Escherichia Castellani and Chalmers 1919. in: Krieg N.R., Holt J.G., (Eds.), Bergey's Manual of Systematic Bacteriology, Vol. 1, Williams & Wilkins, Baltimore, MD, USA, 1984.

[22] Payne W.J., Reduction of nitrogenous oxides by microorganisms, Bacteriol. Rev. 37 (1973) 409–452.

[23] Riedel K.H.J., Britz T.J., Differentiation of 'classical' *Propionibacterium* species by numerical analysis of electrophoretic protein profiles, Syst. Appl. Microbiol. 15 (1992) 567–572.

[24] Riedel K.H.J., Britz T.J., *Propionibacterium* species diversity in anaerobic digesters, Biodiver. Conserv. 2 (1993) 400–411.

[25] Sabaty M., Gans P., Vermèglio A., Inhibition of nitrate reduction by light and oxygen in *Rhodobacter sphaeroides forma* sp. *denitrificans*, Arch. Microbiol. 159 (1993) 153–159.

[26] Schulp J.A., Stouthamer A.H., The influence of oxygen, glucose and nitrate upon the formation of nitrate reductase and the respiratory system in *Bacillus licheniformis*, J. Gen. Microbiol. 64 (1970) 195–203.

[27] Van Gent-Ruijters M.L.W., De Vries W., Stouthamer A.H., Influence of nitrate on fermentation pattern, molar growth yields and synthesis of cytochrome b in *Propionibacterium pentosaceum*, J. Gen. Microbiol. 88 (1975) 36–48.