



## **Effects of Temperature and pH on the Stability of Protease Produced by *Alcaligenes faecalis* P2**

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### **Authors' contributions**

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

### **Article Information**

DOI: 10.9734/JALSI/2021/v24i1130266

### **Open Peer Review History:**

This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: <https://www.sdiarticle5.com/review-history/81087>

**Original Research Article**

**Received 19 October 2021**  
**Accepted 23 December 2021**  
**Published 25 December 2021**

### **ABSTRACT**

**Background:** Bacterial proteases represent a group of very important industrial enzymes. They are involved in the hydrolysis of peptide bond found in protein. Industrial application of bacterial proteases has been limited by low yield and instability at biotechnological process conditions. This study was design to investigate the effect of temperature and pH on the stability of protease produced by *Alcaligene faecalis* strain P2.

**Methodology:** Protease-producing bacteria were isolated from beans effluent-impacted soil and screened for protease production on Casein agar plate. Protease assay was carried out following standard method for protease determination and the stability of the protease produced was investigated over temperature range of 20 to 90 °C and pH range of 3 to 12. The protease-producing bacterium was identified using its molecular characteristics.

**Results:** Protease assay result showed that the amount of tyrosine released by one unit of the crude enzyme was 0.176 µmol/mL resulting in 0.19 U/mL protease activity. Stability studies showed that the protease had wide spectrum of pH and temperature stability. The protease was most stable at pH 9 and temperature of 40 °C after 30 min. Maximum protease activity of 1.259 U/mL was recorded with pH 9 after 30 min. The protease-producing bacterium was classified as *Alcaligene faecalis* P2 based on phylogenetic analysis of its 16S gene analysis. The sequences have been submitted to GenBank under the accession number MZ477004.

**Conclusion:** This study therefore has demonstrated that beans effluent-impacted soil harbours protease producing bacteria with high industrial potentials. In addition, the study revealed that the protease produced in this study can retain its activity over wide temperature and pH.

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**Keywords:** Protease; beans effluent; *Alcaligene faecalis*, casein agar.

## 1. INTRODUCTION

Proteases are commercially important enzymes due to their wide applications in the industry. Proteases occupy the largest part in commercial enzyme market and find applications in many biotechnological processes. A review of industrial application of proteases by Rassaq et al. [1] revealed that proteases have been applied in detergent and leather industries where they help in the removal of blood stains and in wool making [2-7]. They have been used in soy sauce, protein hydrolysate, production of seasoning material, beer clearing and fruit juice, as digestive aids, tendering the fibril muscle, improving texture of flour paste [8,9], in food industry and brewery [1].

Protease can be produced from different sources but the production of proteases from microorganisms have flourished immensely. Interests in microbial proteases have been attributed to the wide applicability of these proteases. Moreover, certain features such as high yield, less production time, less space requirement, easy genetic manipulation, and cost efficiency, have increased their suitability for many biotechnological applications [10,11].

In order to make proteases industrially attractive and competitive, a number of techniques have been employed in improving their yield [1]. Some of these methods used to optimize the production of protease include response surface optimization, exploitation of different substrates and genetic manipulation [12,13]. Moreover, researchers have exploited various techniques with the aim of improving protease yield for industrial applications. Some of such techniques have included cloning and overexpression, fed batch, screening of strains, and chemostat fermentation. Different statistical methods including one factor at a time and response surface methodology have been exploited in the optimization of medium and growth parameter for improved yield [14,15,16,17].

Despite the successes recorded and advantages of using microbial proteases, a number of factors have limited their large-scale industrial applications [1]. Major challenges have been thermal instability coupled with high cost of enzyme recovery; these have restricted the application of proteases in the industry.

This study was therefore designed to investigate the effect of pH and temperature on the stability of protease from *Alcaligenes faecalis* P2 isolated from beans effluent-impacted soil in Port Harcourt, Rivers State, Nigeria.

## 2. RESEARCH METHODOLOGY

### 2.1 Sample Collection

Beans effluent-impacted soils were collected from Rumuosi, Port Harcourt, Rivers State. The soil was air-dried for 24 h, sieved to remove debris and thereafter used for the isolation of protease-producing bacteria.

### 2.2 Serial Dilution, Isolation and Screening for Proteolytic Bacteria

Serial dilution was performed on the soil samples as described by Jalal et al. [18]. Nine (9) millilitres of normal saline (0.85% of NaCl w/v in distilled water) was dispensed into clean test tubes and the test tubes were sterilized in an autoclave at 121°C (15 psi) for 15 min and then allowed to cool. One gram (1 g) of the dry soil sample was dissolved in the 9 ml sterile normal saline to make a stock solution. From this stock solution several ( $10^{-1}$  to  $10^{-6}$ ) dilutions were made.

Protease-producing bacteria were isolated using the medium composition consisting of (g/L): lactose 10; casein 10;  $\text{KH}_2\text{PO}_4$  2;  $\text{K}_2\text{HPO}_4$  2;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  1, and agar 15. One hundred microlitre of aliquots from various dilutions ( $10^{-3}$  -  $10^{-6}$ ) were plated in duplicate on agar. Inoculated agar plates were incubated at ambient temperature for 48 h [19]. Bacteria that showed clearance zones on the agar plates were selected as protease producers and thereafter sub-cultured onto freshly prepared agar plates. These protease producers were purified on nutrient agar plate and store in an agar slant containing the minimal medium.

### 2.3 Protease Assay

Protease assay was carried out according to the method described by Tambekar and Tambekar [20]. In brief, selected protease-producing bacterial isolates were inoculated into 20 mL of protease production medium comprising: lactose 10 g; casein 10 g;  $\text{KH}_2\text{PO}_4$  2 g;  $\text{K}_2\text{HPO}_4$  2 g; and

MgSO<sub>4</sub>.7H<sub>2</sub>O 1 g); the medium was thereafter, incubated for 48 h on a rotary shaker (200 rpm) at room temperature [19,21,20]. After incubation, the culture media were each centrifuged at 5000 rpm for 20 min and at 4°C. Cell-free supernatants were collected and used as the crude enzyme for protease assay.

Protease assay was conducted by a modified method described by Cupp-Enyard [22]. Casein dissolved in different pH buffers [acidic (pH:4), neutral (pH:7) and alkaline (pH:9)] were used as the substrate for the assay. The reaction mixture contained casein and the crude enzyme solution. The mixture was incubated at 37°C for 10 min. Thereafter, the reaction was stopped by addition of 3 ml of 20% ice-cold trichloro acetic acid (TCA). Precipitated proteins were removed by centrifugation and 0.5 ml of the supernatant was mixed with 2.5 ml of 0.5 M Na<sub>2</sub>CO<sub>3</sub> and kept for 20 min at room temperature. Finally, Folin's phenol reagent was added to the mixture, kept for 10 min and absorbance was measured at 660 nm against the blank sample. The amount of enzyme required to liberate 1 μmole of tyrosine per ml per minute under the standard conditions defined one unit of protease activity [23].

#### 2.4 Effect of pH on the Stability of Protease

The stability of the crude protease over wide pH range (3 to 12) was examined. The methods of Nizamudeen and Bajaj [24] and Ponnuswamy and Prakash (2012) were employed to determine the effect of pH on crude protease stability. A volume of 0.5 mL of the crude enzyme was pre-incubated without substrate; thereafter the buffer was adjusted to the appropriate pH values and incubated at 35°C for 15 min, 30 min and 1 h, respectively and the protease activity determined thereafter.

#### 2.5 Effect of Temperature on the Stability of Protease

The stability of the crude protease over wide temperature range (20 to 90°C) was examined. To determine thermal stability of the enzyme, 0.5 mL of the crude protease was incubated without substrate at increasing temperatures of 20, 30, 40, 50, 60, 70 and 90°C for 15 min, 30 min and 1 h, respectively and then assayed for protease activity.

#### 2.6 Identification of Protease-Producing Bacteria

The selected protease-producing bacterium was identified based on the biochemical and molecular characteristics of the isolate. Bacterial genomic DNA extraction was carried out using ZR Soil Microbes DNA Mini-Prep extraction kit (Zymo Research Corporation, South Africa). The quantity and purity of the extracted genomic DNA of the bacterium was analysed using an ND-2000c NanoDrop spectrophotometer (Thermo Scientific, Inqaba Biotech, South Africa) and agarose gel electrophoresis. The extracted genomic DNA was stored at -20°C. The primer sets 27F (5<sup>1</sup>AGAGTTTGATCMTGGCTCAG-3<sup>1</sup>) and 1492R (5<sup>1</sup>GGGTTACCTTGTTACGACTT3<sup>1</sup>) were used for PCR amplification. PCR reaction parameters included: PCR cocktail of volume 25 μL consisting of 12.5 μL of the Master Mix (Zymo Master Mix), 0.4 μL of each primer, 5 μL of the DNA template and 6.7 μL of sterile nuclease-free water. The PCR conditions were as follows: Initial denaturation (95°C for 5 min.), denaturation (95°C for 30 sec.), annealing (52°C for 30 sec), extension (72°C for 45 sec.), final extension step (72°C for 3 min) and hold (4°C). About 5 μL of the amplified products was run on agarose gel electrophoresis at 120 V for 15 min to determine the quality of the products. The amplified products were also purified using DNA clean and concentrator (DCC) kit (Zymo research institute, South Africa) in preparation for sequencing.

PCR products of the bacterial DNA were sequenced with 3500 ABI genetic analyser at Inqaba Biotechnical Industries, South Africa utilizing the Sanger Sequencing technology. Generated sequences were visualized using ChromasLite for base calling. BioEdit was used for sequence editing and Basic Local Alignment Search Tool (BLAST) was performed using NCBI (National Center for Biotechnology Information) database. Similar sequences were downloaded and aligned with ClustalW and Muscle and phylogenetic tree drawn with MEGA X software [25].

### 3. RESULTS AND DISCUSSION

#### 3.1 Screening Characteristics of the Protease-Producing Bacterium

The protease-producing bacterium (*Alcaligena faecalis* P2) used in this study showed zone of

clearance on Casein agar plate. Hydrolysis of the isolate on casein agar plate is shown in Fig. 1. The zone of hydrolysis on casein agar showed 4.16 cm<sup>2</sup> clearance zone. Protease assay result showed that the amount of tyrosine released by one unit of the crude enzyme was 0.176 μmol/mL resulting in 0.19 U/mL protease activity.

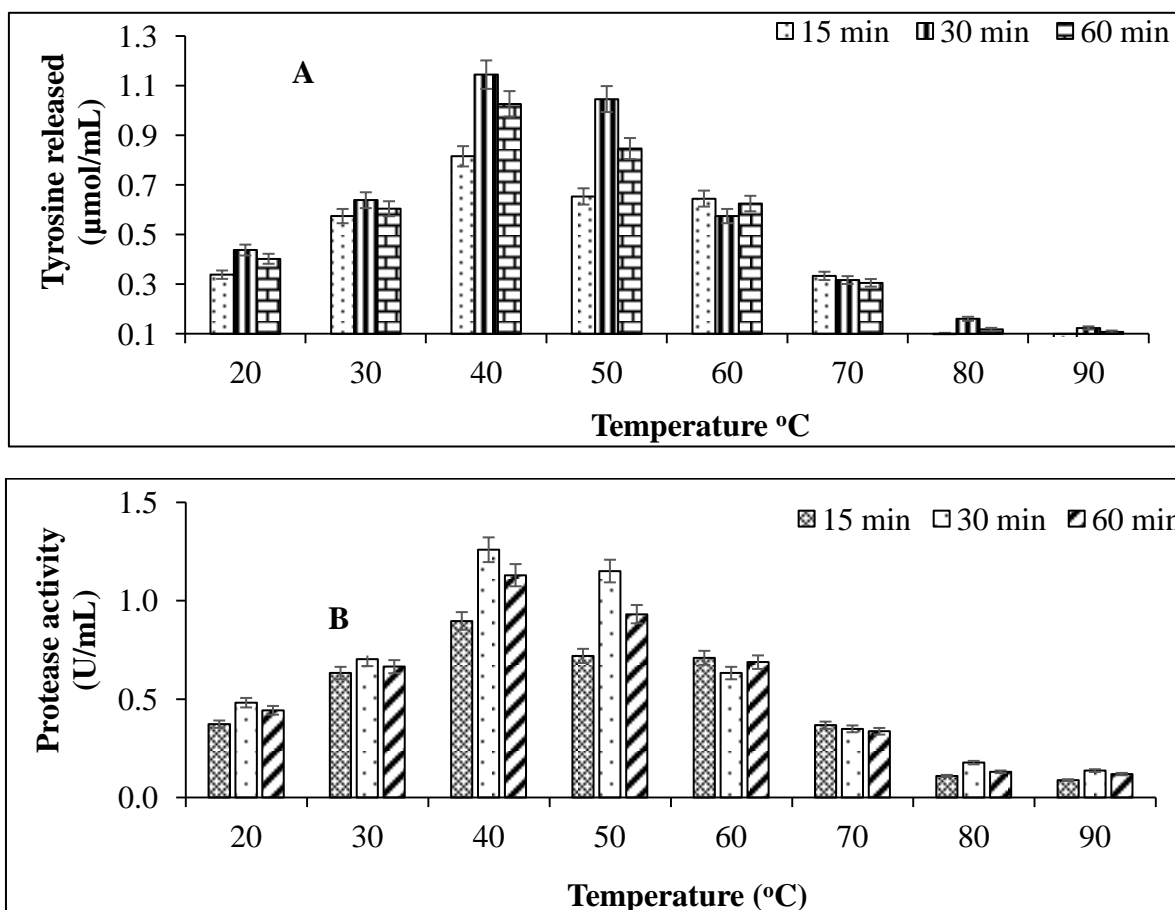
### 3.2 Thermal and pH Stability of the Crude Protease

Data obtained from the stability result showed that the protease was stable over wide pH and temperature ranges. The amount of tyrosine released and protease activity were highest after 30 mins and at temperature of 40°C. Maximum tyrosine was 1.144 μmol/mL whereas the highest protease activity was 1.259 U/mL (Fig. 2). Highest pH stability was observed at pH 9 with corresponding maximum tyrosine released and protease activity as 1.94 μmol/mL and 2.13 U/mL, respectively after 30 min (Fig. 3).

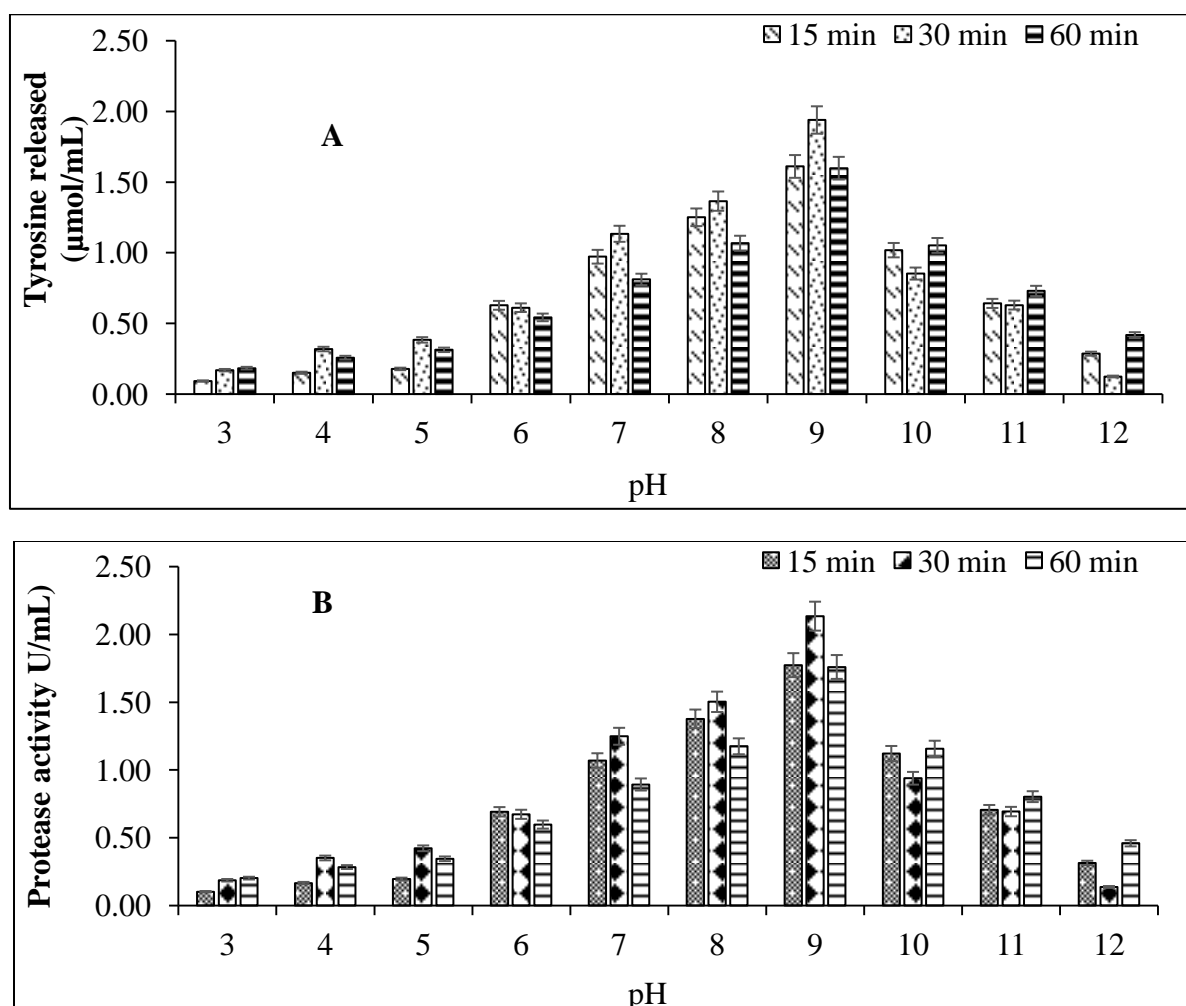


*Alcaligenes faecalis* P2 on Casein agar plate

**Fig. 1. Casein Agar Plate screening of the protease-producing bacterium *Alcaligenes faecalis* P2**



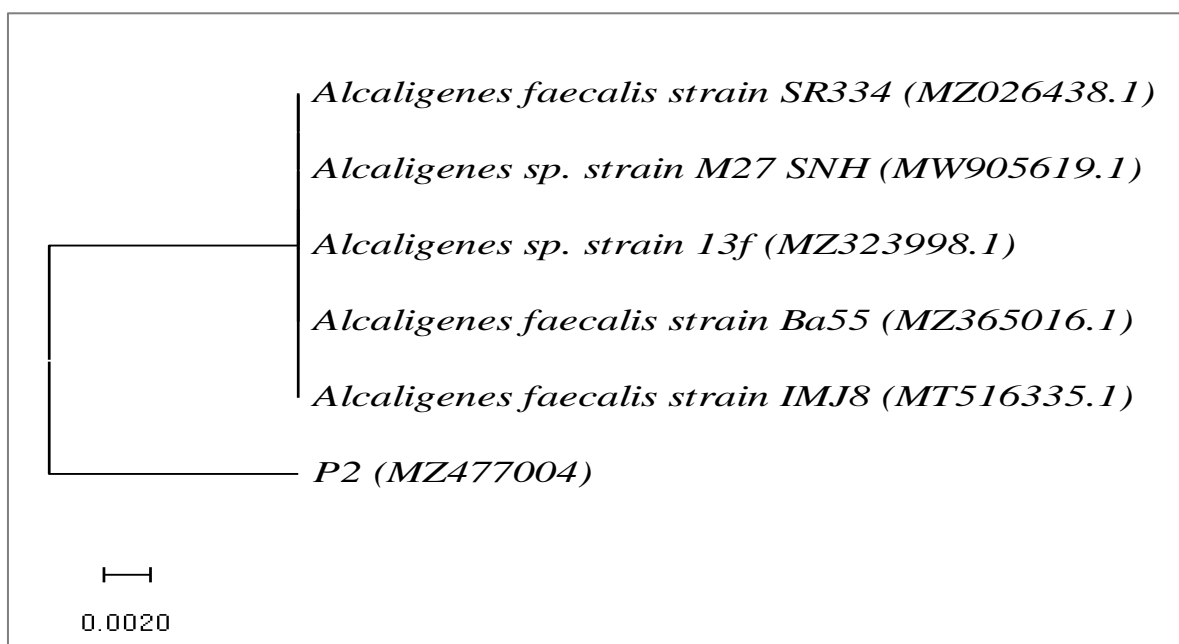
**Fig. 2. Stability of protease produced by *Alcaligenes faecalis* P2 at different temperatures indicated by released tyrosine and protease activity. (A = tyrosine released; B = protease activity). The stability was determined after 15, 30 and 60 minute intervals**



**Fig. 3. Stability of protease produced by *Alcaligenes faecalis* P2 at different pH indicated by released tyrosine and protease activity (A = tyrosine released; B = protease activity). The stability was determined after 15, 30 and 60 minute intervals**

**Table 1. Biochemical characteristics of the protease-producing isolate**

Tests	Characteristics
Gram Staining	Gram negative coccobacilli
Oxidase	+
Catalase	+
Indole	-
Citrate	+
TSI agar (Slant/Butt)	Red/Red
H <sub>2</sub> S production	-
Methyl Red	-
Voges Poskauer	-
Urease	-
Glucose fermentation	-
Lactose fermentation	+
Starch hydrolysis	-
Nitrate production	-
Gelatin hydrolysis	-
Probable Organism	<i>Alcaligenes</i> sp.



**Fig. 4. Phylogenetic tree of protease-producing bacterium *Alcaligenes faecalis* P23.4**

### 3.3 Identification of the Protease-Producing Bacterium

Biochemical characteristics of the bacterial isolate used in this study are given in Table 1. From the table, the isolate was a Gram negative coccobacilli-shaped bacterium. It was not able to ferment glucose but able to ferment lactose.

Phylogenetic tree analysis of the DNA sequences obtained from the protease-producing bacteria, classified the isolate as *Alcaligenes faecalis* P2. The phylogenetic tree showing the related GenBank isolates is presented in Fig. 4.

### 3.4 Discussion

This study investigated the effect of pH and temperature on the stability of protease produced by *Alcaligenes faecalis* P2 isolated from beans effluent-impacted soil from Port Harcourt, Rivers State, Nigeria. Beans effluent is a rich source of protein and was therefore chosen due to its likelihood to harbour microorganisms with proteolytic capacity. Many studies have described the isolation of protease-producing bacteria from different kinds of soils. Luang-In et al. [26] described the isolation of protease-producing bacteria from soil. Udgire et al. [27] reported the isolation of protease-producing bacteria from tannery effluents. This shows that many different environments and effluents can

effectively serve as source of protease-producing bacteria.

The bacterium used in this study was classified as *Alcaligenes faecalis* P2 based on phylogenetic analysis of its 16S gene analysis. The sequences have been submitted to GenBank under the accession number MZ477004. Thangam et al. [28] reported that the *Alcaligenes faecalis* isolated from tannery soil produced protease efficiently. Similarly, *Alcaligenes faecalis* was reported as an efficient alkaline protease producer by Marathe et al. [29]. The findings of this study corroborate this claim made by Marathe et al. [29] as the *Alcaligenes faecalis* strain P2 used in this present study produced protease best at alkaline condition.

The *Alcaligenes faecalis* P2 was stable over a wide range of pH and temperature. This observation is important in industrial application of the enzyme due to variation in temperature and pH conditions during biotechnological processes. Temperature and pHs are rate limiting factors in enzyme activity. Bhunia et al. [30] noted that at higher or lower pHs, the stable 3-D structures of enzymes get compressed, resulting in lower residual activity. The findings of this study corroborated this assertion as at lower and higher temperatures and pH above the optimum, residual protease activity was greatly reduced.

#### 4. CONCLUSION

This study demonstrated that the protease-producing bacterium, *Alcaligenes faecalis* P2 used in this study was stable over wide range of temperature and pH, with the optimum pH at 9 and temperature at 40 °C. The protease produced by the isolate retained activity between temperatures of 20 and 90 °C and between pH of 3 and 12. The stability of enzymes over wide temperature and pH ranges are important characteristics for industrial applications as most industrial applications are performed at varying temperature and pH ranges. Thus, the protease used in this study can be scaled up for industrial application. Furthermore, the study revealed that soils impacted by rich proteineous substrates such as beans effluent are rich source of protease-producing bacteria.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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