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Production of Amino Acid by R. Sulfoviridis Grown on Pineapple Peel Extract at Three pH Values

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Abstract

Organic waste generation in Nigeria is about 68,000tonnes per day. All these are thrown into the environment either untreated or buried in landfills, constituting environmental pollution. Some photosynthetic bacteria can utilize these wastes as source of carbon for growth and Rhodopseudomonas species have been implicated in waste treatment. In an effort to determine if R. sulfoviridis is associated with this hypothesis, 125ml of sodium succinate yeast extract broth supplemented with 0.5mM EDTA was inoculated with 6.25m1 (5%) of stream water collected from Uli town in Anambra State of Nigeria. Cultural characteristics included greenish turbidity in broth culture and branching large greenish colonies on solid agar medium. Motility was positive, Gram stain, negative and sulfide oxidation was sulfate, confirming the isolate to be R. sulfoviridis by Bergey's criteria. Previous studies have shown that R. sulfoviridis grows at pH range of 5.5 to 7.0 therefore ability of R. sulfoviridis to grow in pineapple peel extract without yeast extract and produce amino acid at three pH values, 5.5, 6.8 and 7.0 was monitored using amino acid analyzer, AAI109M002. There was a yield of different quantities of alanine, lysine, glutamic acid, aspartic acid, glycine, leucine, tyrosine, arginine, methionine and cysteine. On day 5, tyrosine was 2.88µg, 2.48µg and 1.38µg at pH 7.0, 6.8 and 5.5 respectively. Pineapple peel and R. sulfoviridis are useful for the actualization of environmental sustainability.

Keywords: Rhodopseudomonas sulfoviridis, Pineapple Peel Extract, Amino acid

Introduction

Most bacteria have the ability to degrade organic carbon compounds either in aerobic or anaerobic conditions with the production of primary metabolites (Agrawal, *et al.,* 2007; Jeong *et al*., 2008; Reungsang *et al.,* 2007) Among these bacteria are the purple non-sulphur photosynthetic bacteria which are metabolically versatile in nature. They can also grow both anaerobically in the light and aerobically in the dark (Kantachote, *et al.,* 2005.) This physiological nature has earned them a wide acceptance for industrial and environmental applications (Madukasi *et al.,*2011; Afsar *et al.,* 2011) Their use for environmental purposes coincides with their industrial importance in the sense that their primary metabolites produced in the process of treating any waste from the environment are usually products of commercial value when purified. Examples of such products which previous studies have shown include hydrogen gas (Jeong *et al.,* 2008; Gadhamshetty *et al*., 2008; Hasson, 2009); volatile fatty acids i.e. acetic, butyric and propionic acids (Sangyoka, *et al.,* 2007; Reungsang *et al.,* 2004) and single cell proteins (Bolliger, *et al.,* 1985). So far, there are no reports on the analysis of the growth culture of photosynthetic bacteria for the presence of such primary metabolites as amino acids, vitamins and nucleotides. It is therefore the aim of this work to investigate the growth culture of *Rhodopseudomonas sulfoviridis* grown in pineapple peel extract at three pH values for the presence of amino acids. The ability of the organism to grow on certain sugars was also investigated, for comparison.

Objectives

1) To isolate the organism, *R. sulfoviridis* by selective enrichment and characterize it by sugar fermentations and sulfate oxidation.
2) To determine the optimum pH for its growth
3) To investigate its ability to grow on pineapple peel extract, acetate, ethanol, fructose, glucose, malate, and succinate.
4) To analyze the culture medium for the presence of amino acids

Materials and Methods

Collection of Samples

The water sample was collected from Ubahudara stream in Uli town, Anambra State, Nigeria. Transparent plastic bottle that was disinfected with 100ppm sodium hypo chloride was used for the collection according to Mahakhan *et al.* (2002). The sampling was done in the dry season and was processed within 24hours of the collection

Isolation of *R. sulfoviridis*

This was done by selective enrichment and also by streaking an agar medium for single colonies. The medium used for this was sodium succinate yeast extract medium according to Lindquist (2000) and modified according to Hougandy *et al.* (2000) by adding 0.5mM EDTA. This according to Kern *et al.* (1992) will strongly inhibit the growth of *Rhodobacter* sp at this concentration. The medium composition is as follows:

This medium composition is in three parts.

1. Basal medium, consisting of KH_2PO_4, 0.33g; $MgSO_4.7H_2O$, 0.33g; $NaCl_2$, 0.33g; NH_4Cl, 0.5g; $CaCl_2.2H_20$, 0.05g; Sodium succinate, 1.0g; and yeast extract, 0.02g.

These were dissolved in 1L of distilled water; pH was 6.8. It was autoclaved at 121°C for 15mins and 15lbs pressure.

2. Trace salts solution consisted of $ZnSO_47H_2O$, 0.01g; $MnCbAH_20$, 0.003g; H_3BO_3, 0.03g; $COCl_2.6H_2O$, 0.02g; $CuCl_2.2H_2O$, 0.01g; $NiCb.6H_20$, 0.002g; and $Na2MoO_4$, 0.003g. These were dissolved in 1L of distilled water; pH was 4.0
3. Sterile solutions added after autoclaving the basal medium above, consisted of 1.0ml of the above mentioned trace salts solution and 0.5ml of 0.02% $FeSO_4.7H_2O$ solution.

Procedure for the Enrichment and Incubation of the culture

The culture bottles used for the enrichment were transparent glass bottles with stopped lids and 125ml in volume. Into each culture bottle, an 80ml volume of enrichment broth was aseptically dispensed and autoclaved at 121°C, 151b pressure for 15mins. Then varying volumes of the water sample (2.5ml, 6.25ml, 8.75ml, 12.5ml and 18.7Sml) representing 2%, 5%, 7%, 10% and 15% respectively, of the 125ml volume of the culture bottle, were dispensed into each culture bottle, containing the broth medium. For each percentage, a triplicate number of bottles were prepared. After this inoculation, each of the culture bottles was filled to the neck of the bottle with the broth medium in order to ensure that air bubbles are not trapped in the culture bottle. Some sterile Vaseline oil was placed on top of the culture medium in the culture bottles before they were covered with both foil paper and paper tape. This was to create anaerobic condition within the culture bottle according to Kantachote *et al.,* (2005). All the culture bottles were properly labeled for ease of identification and then placed on the laboratory bench to incubate at room temperature under illumination by 150W tungsten lamp, placed at a distance of 90cm from the culture bottles.

Streaking for pure isolates

The medium used is the same for the enrichment, but 1.5g agar – agar was added to solidify it for the streaking. The addition of agar - agar altered the pH of the medium which was 6.8 originally. However, it was readjusted by addition of 0.1ml Na0H solution to the required pH of 6.8, as above. The medium was then sterilized by autoclaving at 121°C, 15lb pressure for 15mins. After cooling about 20ml of the medium was dispensed into sterile Petri dishes and allowed to solidify. Sterile Vaseline oil was aseptically poured into the covers of each of the Petri dishes and allowed to solidify before being used to cover the plates. Cultures from the liquid enrichments were used for this streaking and afterwards, the plates were incubated on the laboratory benches at room temperature under illumination by 150W tungsten lamp placed at a distance of 90cm from the plates. The edges of the plates were also sealed with Vaseline oil and paper tapes.

Characterization of the Isolate

Using Manufacturer's instruction , 4.2g of nutrient broth, 2g of the indicator, 10g of each of the sugars and 10g of each salt, were separately dissolved in 100ml of distilled water. Later, they were sterilized by autoclaving at 121° C at 15Ib pressure for 15mins. After cooling, 1ml of each of the sterilized solutions was transferred into the test tubes containing the organism and these tubes were incubated under a light source for 48 – 72 hours at room temperature.

Growth of *R. sulfoviridis* on acetate, ethanol, fructose, glucose, malate, succinate, and pineapple peel extract at pH range 5.5, 6.8, and 7.0

One Gram of each of the carbon sources was measured and separately diluted in 41.4ml of sterile water. 1ml of peptone water and 0.5ml of KH2PO4 were also dispensed into the mixture. Also three drops of phenol red were added into it. This mixture was prepared in three sets each for one pH value being investigated, and for each range, triplicate test tubes were inoculated with the organism for the experiment. Durham tube was placed into each in an inverted position. Then the tubes were covered with cotton wool and incubated at room temperature under tungsten lamp for 14 days and monitored for gas production.

Growth medium for amino acid determination

The medium used to grow R. sulfoviridis for the determination of amino acid production was a modified version of the medium used for investigating its ability to grow. The only carbon source was pineapple peel extract and no yeast extract was added. The incubation condition was the same. At the end of the incubation, the medium was analyzed for amino acid presence using amino acid analyzer with serial number AA1109M002 done by Anima service Consult Nigeria, Ltd, Port Harcourt, Rivers State.

Results

Isolation and Characterization of the *R sulfoviridis.*

The cultural morphology showed that the organism is greenish in color. The Gram stain showed that the organism was Gram negative, the wet mount showed that the organism is motile. Sugar fermentation tests results and the phenotypic characteristics of the organism are presented on Table 1

Table 1 Phenotypic Characterization and Identification

Teats	Reactions
Color of culture	Green
Gram staining	-
Wet mount	Motile
Aerobic dark growth	+
Nitrate utilization	+
Sulfate utilization	+
Fermentation tests	
Glucose	-
Malate	+
Succinate	+
Fructose	+
Ethanol	-
Acetate	+

Estimation of optimum pH and sugar of best support for growth by turbidity and pigmentation

Table 2 shows the growth mode of *R. sulfoviridis* on acetate, ethanol, fructose, glucose, malate, succinate, and pineapple peel at pH 5.5. From days 1 to 6, no evidence of growth was observed. The first evidence of growth was recorded on day 7 and the growth ceased on day 14. Except glucose and ethanol which did not support the growth, the effect of the rest of the sugars and pineapple peel was the same.

Table 3 shows the growth mode of *R. sulfoviridis* on acetate, ethanol, fructose, glucose, malate, succinate, and pineapple peel at pH 6.8. From days 1 to 4, no evidence of growth was observed. The first evidence of growth by turbidity was recorded on day 5 while pigmentation was observed on day 6. The growth ceased on day 14. Glucose and ethanol did not support the growth but the rest of the sugars, and pineapple peel supported in equal mode.

Table 4.4 shows the growth mode of *R. sulfoviridis* on acetate, ethanol, fructose, glucose, malate, succinate, and pineapple peel at pH 7.0: from days 1 to 5, no evidence of growth was observed. The first evidence of growth by pigmentation and turbidity was recorded on day 6 and the growth ceased on day 14. Glucose and ethanol did not support the growth but the other sugars and pineapple peel did, in the same pattern.

Table 2: The different Carbon sources at pH 5.5

	Glucose		**Malate**		**Succinate**		**Fructose**		**Ethanol**		**Acetate**		**Pineapple peel**	
Day	**T**	**P**	**T**	**P**	**T**	**P**	**T**	**P**	**T**	**P**	**T**	**P**	**T**	**P**
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	+	+	+	+	+	+	-	-	+	+	+	+
8	-	-	+	+	+	+	+	+	-	-	+	+	+	+
9	-	-	+	+	+	+	+	+	-	-	+	+	+	+
10	-	-	+	+	+	+	+	+	-	-	+	+	+	+
11	-	-	+	+	+	+	+	+	-	-	+	+	+	+
12	-	-	+	+	+	+	+	+	-	-	+	+	+	+
13	-	-	++	++	++	++	++	++	-	-	++	++	++	++
14	-	-	++	++	++	++	++	++	-	-	++	++	++	++

Turbidity –T
Pigmentation – P
Absence of turbidity and pigmentation –
Presence of turbidity and pigmentation +
Increase in turbidity and pigmentation ++

Table 3: The different Carbon sources at pH 6.8

Glucose	**Malate**	**Succinate**	**Fructose**	**Ethanol**	**Acetate**	**Pineapple**

													peel	
Day	T	P	T	P	T	P	T	P	T	P	T	P	T	P
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	+	-	+	-	+	-	-	-	+	-	+	-
6	-	-	+	+	+	+	+	+	-	-	+	+	+	+
7	-	-	+	+	+	+	+	+	-	-	+	+	+	+
8	-	-	+	+	+	+	+	+	-	-	+	+	+	+
9	-	-	+	+	+	+	+	+	-	-	+	+	+	+
10	-	-	+	+	+	+	+	+	-	-	+	+	+	+
11	-	-	+	+	+	+	+	+	-	-	+	+	+	+
12	-	-	+	+	+	+	+	+	-	-	+	+	+	+
13	-	-	++	++	++	++	++	++	-	-	++	++	++	++
14	-	-	++	++	++	++	++	++	-	-	++	++	++	++

Turbidity –T
Pigmentation – P
Absence of turbidity and pigmentation –
Presence of turbidity and pigmentation +
Increase in turbidity and pigmentation ++

Table 4: The different Carbon sources at pH 7.0

	Glucose		Malate		Succinate		Fructose		Ethanol		Acetate		Pineapple peel	
Day	T	P	T	P	T	P	T	P	T	P	T	P	T	P
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	+	+	+	+	+	+	-	-	+	+	+	+
7	-	-	+	+	+	+	+	+	-	-	+	+	+	+
8	-	-	+	+	+	+	+	+	-	-	+	+	+	+
9	-	-	+	+	+	+	+	+	-	-	+	+	+	+
10	-	-	+	+	+	+	+	+	-	-	+	+	+	+
11	-	-	+	+	+	+	+	+	-	-	+	+	+	+
12	-	-	+	+	+	+	+	+	-	-	+	+	+	+
13	-	-	++	++	++	++	++	++	-	-	++	++	++	++
14	-	-	++	++	++	++	++	++	-	-	++	++	++	++

Turbidity –T
Pigmentation – P
Absence of turbidity and pigmentation –
Presence of turbidity and pigmentation +
Increase in turbidity and pigmentation ++

Analysis of the pineapple peel, *R. sulfoviridis* medium for the presence of amino acid.

At pH 5.5, production of amino acids started on day 4 and continued till day 6. The amino acids produced are alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, leucine, lysine, methionine and tyrosine. On that day 4, the percentage of the individual amino acids are 10%, 2%, 8%, 1%, 8%, 7%, 6%, 9%, 1%, and 4% for alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, leucine, lysine, methionine, and tyrosine respectively. On day 5, the percentage production for the respective amino acids was 10%, 4%, 8%, 1%, 8%, 7%, 7%, 11%, 3%, and 4%. On day 6, the yield was 10%, 2%, 9%, 2%, 9%, 9%, 7%, 8%, 2% and 4% for the respective amino acid, fig 1.

At pH 6.8, production commenced on day 4 and continued till day 6. Alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, leucine, lysine, methionine and tyrosine were produced and percentage of each on that day was 10%, 3%, 7%, 1%, 7%, 7%, 6%, 9%, 1% and 3% respectively. On day 5, the amount had increased to 9%, 3%, 11%, 1%, 9%, 7%, 10%, 9%, 2% and 4% for the respective amino acids. On day 6, the record obtained was 11%, 3%, 9%, 2%, 9%, 7%, 10%, 2% and 6% respectively, fig 2.

At pH 7.0, production began on day 4 and stopped on day 6. Alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, leucine, lysine, methionine and tyrosine were produced and the percentage of each on that day was 12%, 2%, 8%, 1%, 8%, 7%, 6%, 10%, 1% and 3%. On day 5, the amounts obtained were 9%, 5%, 12%, 1%, 10%, 7%, 10%, 7%, 3% and 6% for the respective amino acids. On day 6, yield was 12%, 3%, 10%, 1%, 10%, 8%, 7%, 12%, 3%, and 5% respectively for each of the amino acids, fig 3.

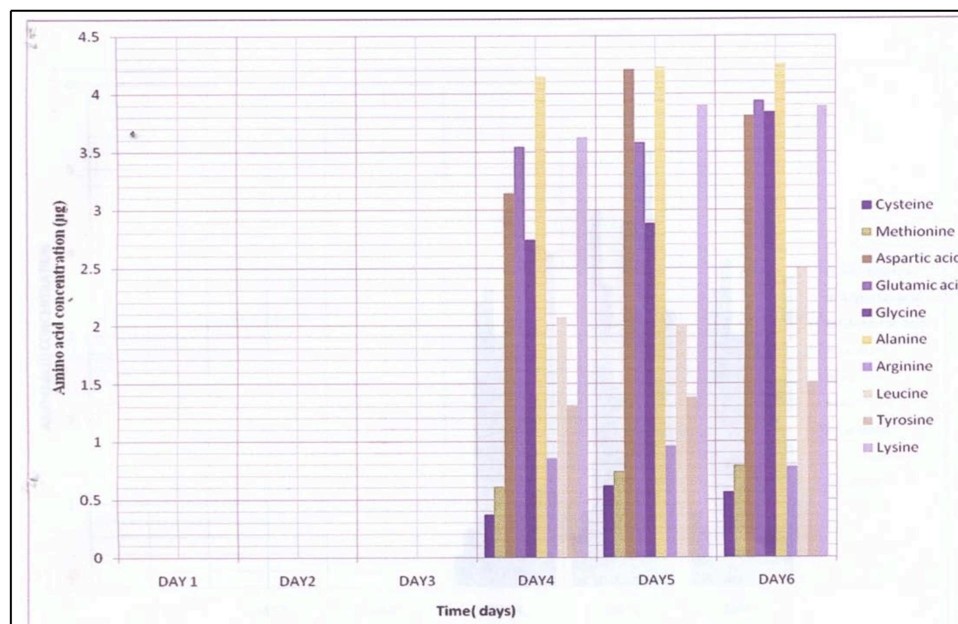

Fig 1: Amino acid production of *Rhodopseudomonas* sp in pineapple peel on bases of time at pH 5.5

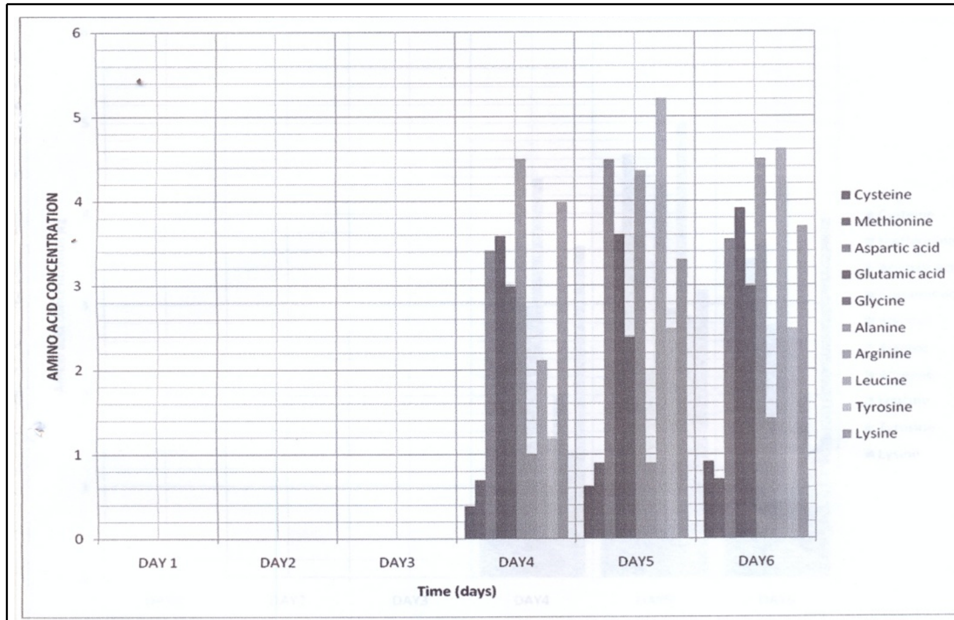

Fig 2: Amino acid production of *Rhodopseudomonas* sp in pineapple peel on bases of time at pH 6.8

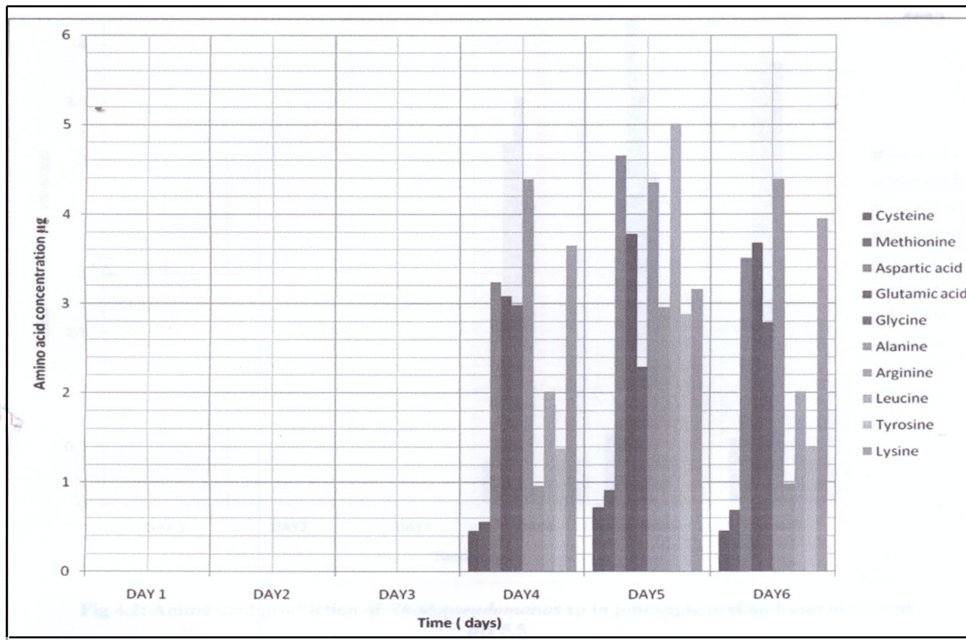

Fig 3: Amino acid production of *Rhodopseudomonas* sp in pineapple peel on bases of time at pH 7.0

Discussion

The form of selective enrichment used for the isolation of *R. sulfoviridis* was very effective according to Lindqunst (2005). The culture obtained was not a mixed culture of organisms, but a pure greenish bloom of spreading colonies whose Gram reaction

was Gram negative. The wet mount showed motility and the organism exhibited positive for acetate, fructose, malate and succrunete but negative for ethanol and glucose. Both aerobic dark growth, nitrate and sulfate utilization were positive according to Bergey's manual of determinative bacteriology (1995).

The three pH values investigated supported the growth of *R. sulfoviridis,* Tables 2, 3 and 4. But growth was faster on pH 6.8 with a difference of two days for pH 5.5 and a difference of one day for pH 7.0. However, on pH 6.8, pigmentation was observed later on day 6 while turbidity was observed on day 5. This could be as a result of insufficient light supply since light switches off most often. On both pH 5.5 and 7.0, pigmentation and turbidity were recorded on the same days.

The sugars that supported the growth of the organism were acetate, fructose, malate and succinate. This confirms anaerobic photoheterophy (Kern, 1992). Pineapple peel extract also supported its growth in the same measure that the sugars did. Therefore pineapple is as good as those sugars for the growth of *R. sulfoviridis*. However, further investigations would have to confirm these facts. Pineapple peel is one of the waste substances thrown away into our streets, which constitute environment pollution. Its use for the growth of *R. sulfoviridis* will suggest a solution to that problem. Glucose and ethanol did not support the growth Tables 2, 3, and 4.

The amino acids that were detected in the medium were alanine, arginine, aspartic acid, cysteine, glutanic acid, glycine, leucine, lysine, methionine and tyrosin. Pineapple peel extract was the only source of carbon in this medium and yeast extract was not included in this medium. So, these amino acids detected were completely primary metabolites of *R. sulfoviridis* growing on pineapple extract. The presence of other metabolites was not investigated but our results clearly indicate that these amino acids were present in the culture medium after the incubation. In another unpublished work, nitrogen was detected in the medium in which this organism was grown.

Ammonium chloride NH_4Cl_2 was a component of this medium. Under anaerobic conditions, some bacteria carry out denitrification process, converting ammonium to nitrogen gas. Since this experiment was conducted under anaerobic conditions, we suggest that *R. sulfoviridis* is among the group of bacteria that carry out denitrification. In view of this result, we propose that further investigation on the ability of *R. sulfoviridis* to produce amino acids while growing on pineapple peel extract without yeast extract should be carried out.

Conclusion

This preliminary study proved the relevance of *R. sulfoviridis* to environmental sustainability and industrial importance. This is because it grew in pineapple peel extract in a similar manner that it grew on other sugars studied. The presence of amino acids was also detected in the culture medium to which no yeast extract was added.

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