**The Predominance of *Bacillus* species and non-culturable microaerophilic/anaerobic microflora in the aerobically operated predigester of *Nisargruna* biogas plant**

**Running title**: Microbiological analysis of predigester flora

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# Table 1 Colony characteristics of cultures isolated from predigester 1

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Isolate** | **Size** | **Shape** | **Colour** | **Margin** | **Opacity** | **Elevation** | **Consistency** | **Gram nature** | **Arrangement** | **Spore characters and position** |
| **PI1** | 2mm | Circular | White | Uneven | Opaque | Raised with  central depression | Moist | Gram positive  Rods with parallel sides and rounded ends | Mostly singles, some are in pairs | Bulging elliptical  Central and sub-terminal spores |
| **PI2** | 4mm | Irregular | White | Uneven | Semi-transparent | Raised | Moist | Gram positive thick  Rods | Mostly singles, some are in pairs | Non-bulging, oval, central spores |
| **PI3** | 4mm | Circular | Dirty white | Uneven | Semi-transparent | Raised | Moist | Gram positive thin slender rods | Mostly singles, some are in short chains | Non-bulging, oval, central spores |
| **PI4** | 5mm | Irregular | Water-like | Uneven | Semi-transparent | Flat | Gelatinous | Gram positive medium size rods | Singles | Bulging oval,  Central and sub-terminal spores |
| **PI5** | 6mm | Circular | White | Even | Opaque | Raised | Moist | Gram positive big  Rods with parallel sides and square ends | Very long chains | Non-bulging, large, oval, central spores |
| **PI6** | 3-4mm | Circular | Dirty white | Uneven | Opaque | Flat with raised  Margin | Moist | Gram positive medium size rods | Singles, pairs and bundles | Non-bulging, oval, central spores |
| **PI7** | 5-6mm | Circular | Water-like | Even | Semi-transparent | Flat | Mucoid | Gram positive medium size rods, slightly thick | Singles and pairs | Oval and spherical, small, central and sub-terminal spores |
| **PI8** | 6mm | Circular | Dirty white | Uneven | Semi-transparent | Raised with  central depression | Mucoid | Gram positive long rods with parallel sides | Singles and pairs | Oval, bulging, sub-terminal spores |
| **PI9** | 3mm | Circular | White | Uneven | Opaque | Flat with raised  ring at the center | Sticky | Gram positive medium size rods | Singles | Oval, bulging, sub-terminal spores |
| **PI10** | 6-7mm | Circular | Dirty white | Uneven | Opaque | Raised | Moist | Gram positive long thick rods | Singles and pairs | Oval, big spores [occupy 2/3 of cell] Non-bulging, terminal |
| **PI11** | 5mm | Irregular | Dirty white | Uneven | Opaque | Raised with  central thin area | Moist | Gram positive medium size rods | Singles | Oval, bulging, sub-terminal spores |
| **PI12** | 3-4mm | Circular | Dirty white | Even | Opaque | Convex with  crumpled surface | Moist | Gram positive stumpy rods | Singles | Oval, non-bulging, sub-terminal & central spores |
| **PI13** | 5-6mm | Circular | Yellowish white | Uneven | Opaque | Flat with raised margin | Sticky | Gram positive slender rods | Singles | Oval, non-bulging, & central spores |
| **PI14** | 4mm | Circular | White | Uneven | Opaque | Flat with  raised center margin | Sticky | Gram positive medium size rods | Singles and pairs | Oval, non-bulging, & central spores |
| **PI15** | 4mm | Circular | Dirty white | Uneven | Semi-transparent | Raised with  folds on surface | Sticky | Gram positive medium size rods | Singles | Oval, sub-terminal spores |
| **PI16** | 5mm | Circular | Dirty white | Uneven | Semi-transparent | Flat with  raised center | Sticky | Gram positive slender rods | Singles and pairs | Oval, sub-terminal, bulging spores |
| **PI17** | 6mm | Irregular | Water-like | Uneven | Semi-transparent | Raised | Sticky | Gram positive slender rods | Singles | Oval, non-bulging & small spores |
| **PI18** | 3mm | Circular | White | Uneven | Semi-transparent | Raised with  crumpled surface | Mucoid | Gram positive long rods | Singles | Oval, sub-terminal, bulging spores |
| **PI19** | 3-4mm | Circular | White | Uneven | Semi-transparent | Star shaped folds | Mucoid | Gram positive short rods | Singles | Oval, sub-terminal, bulging spores |
| **PI20** | 4mm | Irregular | Water like | Uneven | Semi-transparent | Convex with folds | Mucoid | Gram positive medium size rods | Singles | Oval, central, bulging spores |

# Table 2 Colony characteristics of cultures isolated from predigester 2

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Isolate** | **Size ‘mm’** | **Shape** | **Colour** | **Margin** | **Opacity** | **Elevation** | **Consistency** | **Gram nature** | **Arrangement** | **Spore characters and position** |
| **PII1** | 2 | Irregular | Water-like | Uneven | Opaque | Flat with water-like droplets | Moist | Gram positive medium size rods | Mostly singles | Slightly bulging, oval  central spores |
| **PII2** | 5 | Irregular | White | Uneven | Opaque | Flat with wavy margin | Moist | Gram positive medium size thick rods | Mostly singles | Non-bulging, oval, central spores |
| **PII3** | 5 | Circular | Bright white | Uneven | Opaque | Flat with raised center | Moist | Gram positive stumpy rods | Mostly singles, some are in pairs | Non-bulging, oval, central spores |
| **PII4** | 6 | Irregular | Off-white | Uneven | Opaque [shining] | Flat | Moist | Gram positive medium to long rods | Singles, some are in pairs | Bulging oval,  Central spores |
| **PII5** | 6 | Circular | White | Even | Opaque | Flat | Moist | Gram positive long thick rods | Singles | Bulging, large, oval, central spores |
| **PII6** | 6 | Circular | White | Even | Opaque | Flat | Moist | Gram positive very thick rods with parallel sides and flat ends | Singles and short chains | Non-bulging, elliptical, central spores |
| **PII7** | 6 | Circular | White | Even | Opaque | Flat with raised center | Moist | Gram positive thick rods | Singles and chains | Oval, non-bulging and sub-terminal spores |
| **PII8** | 4 | Irregular | Bright white | Uneven | Opaque | Flat with  raised margin | Moist | Gram positive very thin rods | Singles and chains | Circular, on- bulging, sub-terminal spores |
| **PII9** | 4 | Circular | Light yellow | Uneven | Opaque | Flat | Moist | Gram positive long rods | Singles and pairs | Oval, non-bulging, central spores |
| **PII10** | 5 | Irregular | Bright white | Uneven | Opaque | Flat with lots of fold on it | Moist | Gram positive short to medium size rods | Chains and pairs | Oval, bulging, sub-terminal spores |
| **PII11** | 3 | Irregular | Bright white | Uneven | Opaque | High convex [balloon-like] | Moist | Gram positive thin &short rods | Singles and chains | Oval, non-bulging, central spores |
| **PII12** | 7 | Irregular | Dirty white | Even | Opaque | Flat | Moist | Gram positive very thick rods with parallel sides and flat ends | Singles and pairs | Oval, non-bulging, central spores |
| **PII13** | 4 | Circular | Pale Yellow | Even | Semi-transparent | Flat | Moist | Gram positive medium size rods | Singles | Spherical, non-bulging, & central spores |
| **PII14** | 4 | Circular | Lemon Yellow | Even | Opaque | Flat | Moist | Gram positive short rods | Singles and pairs | Oval, non-bulging, & central spores |
| **PII15** | 4 | Irregular | Water-like | Uneven | Semi-transparent | Raised with  folds on surface | Moist | Gram positive medium size rods | Singles | Oval, sub-terminal central spores |
| **PII16** | 3 | Circular | White | Even | Opaque | Low convex | Moist | Gram positive medium size rods | Singles and pairs | Oval, sub-terminal, bulging spores |
| **PII17** | 10 | Circular | White | Uneven | Opaque | Raised | Moist | Gram positive thick rods | Singles and chains | Circular, bulging & sub-terminal spores |
| **PII18** | 15 | Irregular | Dirty white | Uneven | Opaque | Raised | Moist | Gram positive thick rods, parallel sides and rounded ends | Singles | Oval, sub-terminal, bulging spores |
| **PII19** | 15 | Irregular | Dirty white | Uneven | Opaque | Raised with many folds | Moist | Gram positive long, thick rods | Singles | Oval, sub-terminal, slightly bulging spores |
| **PII20** | 15 | Irregular | Dirty white | Uneven | Opaque | Raised with water like droplets | Moist | Gram positive thin medium size rods | Singles [Y,V shaped] | Oval, terminal, bulging spores |
| **PII21** | 3 | Circular | Dirty white | Uneven | Opaque | Convex | Moist | Gram positive thick rods with rounded ends | Chains | Oval, central spores |
| **PII22** | 4 | Circular | Lemon Yellow | Even | Opaque | Flat with raised border | Moist | Gram positive thick rods | Chains | Oval, bulging central spores |
| **PII23** | 4 | Circular | Bright white | Uneven | Opaque | Convex with hollow center | Moist | Gram positive long to medium size rods | Singles | Oval, bulging central spores |
| **PII24** | 5 | Circular | Dirty white | Uneven | Opaque | Convex with many folds | Moist | Gram positive long rods | Singles | Oval, sub-terminal, slightly bulging spores |

**Table 3** Biochemical tests results of the isolates from predigester1

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Culture**  **No.** | **Tests** | | | | | | | | | | | | | **Identification** |
| **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **K** | **L** | **M** |
| **PI1** | + | + | - | + | + | - | + | - | + | - | - | 6.35 | + | *B. subtilis* |
| **PI2** | + | + | + | - | + | - | + | - | + | - | + | 5.55 | - | *B. cereus* |
| **PI3** | + | + | - | - | + | - | + | - | - | - | - | 5.45 | + | *B. pumilus* |
| **PI4** | + | + | + | + | + | - | + | - | + | - | - | 5.74 | + | *B. licheniformis* |
| **PI5** | + | - | + | - | + | - | + | - | - | - | - | 5.14 | + | *B. laterosporus* |
| **PI6** | + | + | - | + | + | - | - | - | - | - | - | 5.31 | + | *B. pumilus* |
| **PI7** | + | + | - | + | + | - | + | - | + | - | - | 5.72 | + | *B. subtilis* |
| **PI8** | + | + | - | + | + | - | + | - | + | - | - | 5.76 | + | *B. subtilis* |
| **PI9** | + | + | - | + | + | - | + | - | + | - | - | 5.83 | + | *B. subtilis* |
| **PI10** | + | + | + | - | + | - | + | - | - | - | + | 5.13 | + | *B. cereus* |
| **PI11** | + | + | - | + | + | - | + | - | + | - | - | 6.10 | + | *B. subtilis* |
| **PI12** | + | + | - | + | + | - | + | - | + | - | - | 5.98 | - | *B. subtilis* |
| **PI13** | + | + | - | + | + | - | + | - | - | - | - | 6.35 | + | *B. pumilus* |
| **PI14** | + | + | - | + | + | - | + | - | + | - | - | 5.98 | + | *B. subtilis* |
| **PI15** | + | + | - | + | + | - | + | - | + | - | - | 6.10 | + | *B. subtilis* |
| **PI16** | + | + | - | + | - | - | - | - | - | - | - | 5.39 | + | *B. pumilus* |
| **PI17** | + | + | - | + | - | - | + | - | - | - | - | 5.43 | + | *B. pumilus* |
| **PI18** | + | + | - | + | + | - | + | - | + | - | - | 6.31 | + | *B. subtilis* |
| **PI19** | + | + | - | + | - | - | + | - | + | - | - | 6.16 | + | *B. subtilis* |
| **PI20** | + | + | - | + | + | - | + | - | + | - | - | 6.09 | + | *B. pumilus* |

**Note: +** indicates growth/positive result, - indicates no growth/negative result.

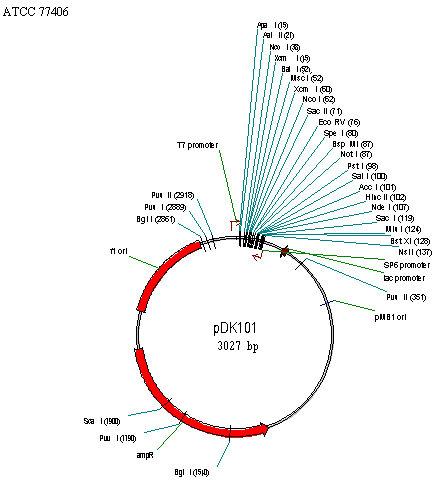
**A**-Catalase test, **B**-Voges Proskauer test, **C**- Growth in anaerobic agar, **D**- Growth at 50°C, **E**-Growth in 7% NaCl, **F**- Acid and gas from glucose, **G**- Nitrate reduction test, **H**-Parasporal bodies, **I**- Hydrolysis of starch, **J**-Growth at 65°C, **K**-Rod breadth 1μ or more, **L**-pH in VP broth <6.0, **M**-Hydrolysis of casein

**Table 4** Biochemical tests results of the isolates from predigester 2

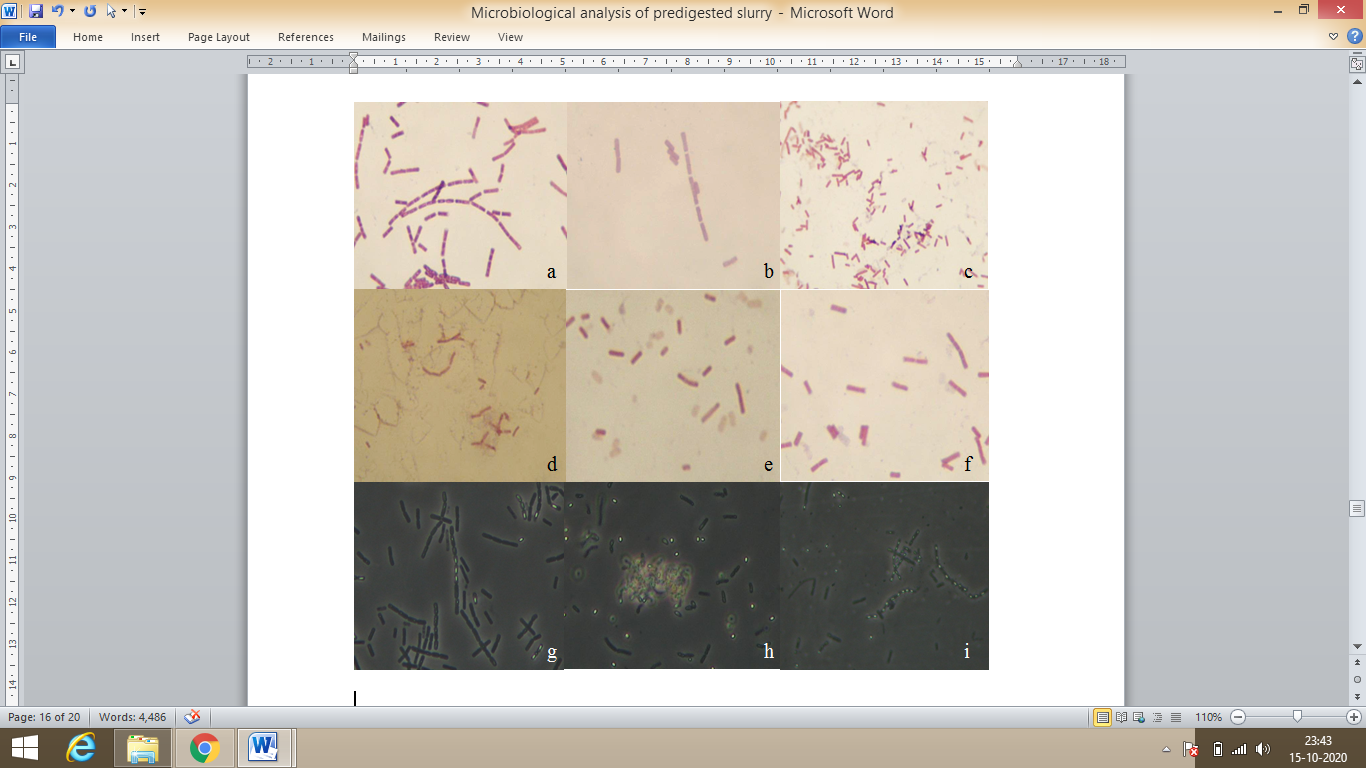
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Culture No.** | **Tests** | | | | | | | | | | | | | **Identification** |
| **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **K** | **L** | **M** |
| **PII1** | + | + | + | + | + | - | + | - | - | - | - | 5.82 | + | *B. licheniformis* |
| **PII2** | + | + | - | - | - | - | + | - | + | - | - | 5.85 | + | *B. subtilis* |
| **PII3** | + | + | - | + | + | - | - | - | + | - | - | 7.88 | - | *B. subtilis* |
| **PII4** | + | - | - | - | + | - | + | - | + | - | - | 5.20 | + | *B.firmus* |
| **PII5** | + | + | + | - | + | - | + | + | + | - | + | 5.27 | + | *B. thuringenesis* |
| **PII6** | + | + | + | - | + | - | + | - | - | - | + | 5.18 | + | *B. cereus* |
| **PII7** | + | + | + | - | - | - | + | - | - | - | + | 5.33 | + | *B. cereus* |
| **PII8** | + | + | - | + | + | - | + | - | + | - | - | 5.87 | + | *B. subtilis* |
| **PII9** | + | + | - | + | + | - | - | - | - | - | - | 5.28 | + | *B. pumilus* |
| **PII10** | + | + | - | + | + | - | + | - | + | - | - | 7.24 | + | *B. subtilis* |
| **PII11** | + | + | - | + | + | - | + | - | + | - | - | 6.69 | + | *B. subtilis* |
| **PII12** | + | + | + | - | + | - | + | - | + | - | + | 5.05 | + | *B. cereus* |
| **PII13** | + | - | - | - | - | - | - | - | - | - | - | 7.08 | - | *B. sphaericus* |
| **PII14** | + | + | - | - | + | - | - | - | - | - | - | 6.14 | + | *B. pumilus* |
| **PII15** | + | + | + | + | + | - | + | - |  | - | - | 6.99 | + | *B. licheniformis* |
| **PII16** | + | + | - | - | + | - | + | - | + | - | - | 5.89 | - | *B. subtilis* |
| **PII17** | + | + | + | - | + | - | + | + | - | - | - | 5.33 | + | *B. thuringenesis* |
| **PII18** | + | + | + | + | - | - | + | + | - | - | + | 5.22 | + | *B. thuringenesis* |
| **PII19** | + | + | + | + | + | - | + | - | - | - | - | 6.51 | + | *B. licheniformis* |
| **PII20** | + | + | + | + | + | - | + | - | - | - | - | 5.21 | + | *B. licheniformis* |
| **PII21** | + | + | - | + | + | - | + | - | + | + | - | 6.47 | + | *B. subtilis* |
| **PII22** | + | - | - | + | - | - | - | - | + | - | + | 5.31 | + | *B. megaterium* |
| **PII23** | + | + | - | + | - | - | + | - | + | - | - | 6.13 | + | *B. subtilis* |
| **PII24** | + | + | - | + | + | - | + | - | + | - | - | 6.05 | + | *B. subtilis* |

**Note: +** indicates growth/positive result, - indicates no growth/negative result.

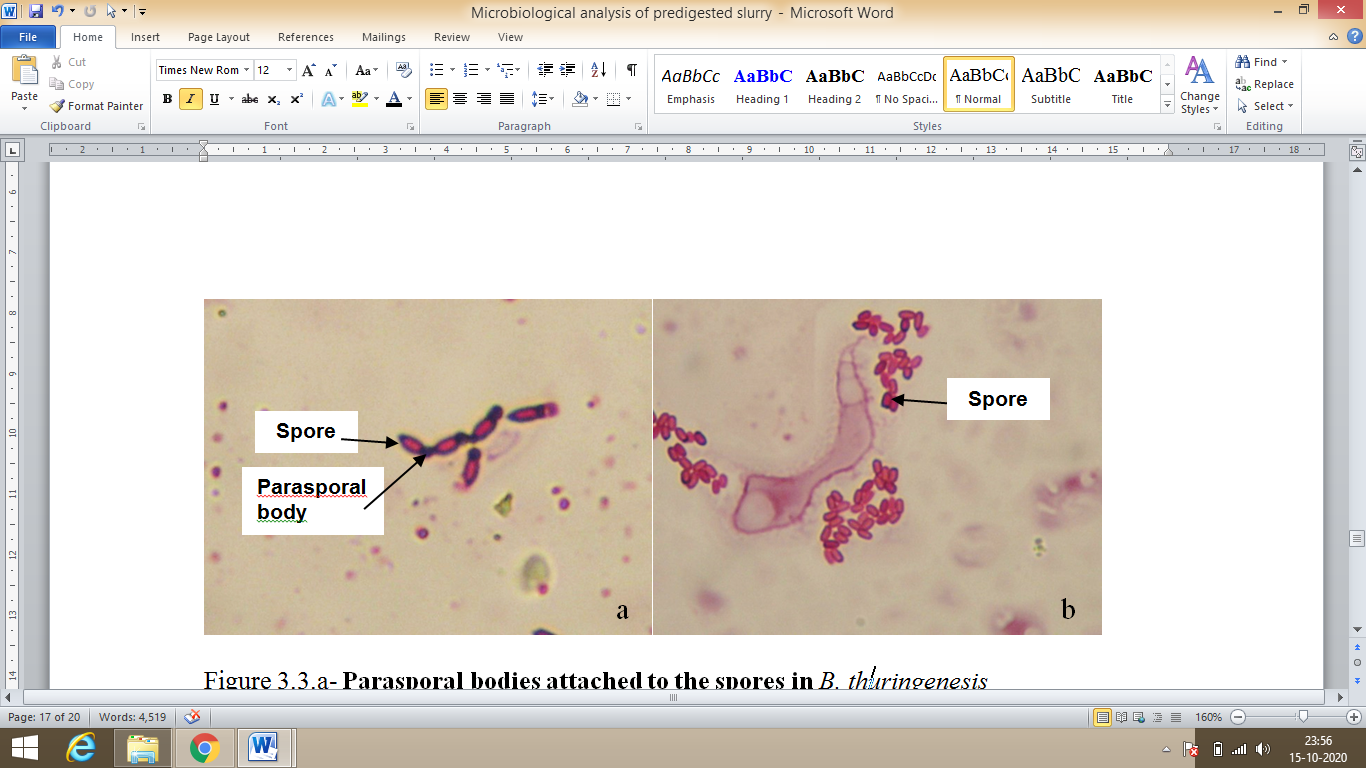
**A**-Catalase test, **B**-Voges Proskauer test, **C**- Growth in anaerobic agar, **D**- Growth at 50°C, **E**-Growth in 7% NaCl, **F**- Acid and gas from glucose, **G**- Nitrate reduction test, **H**-Parasporal bodies, **I**- Hydrolysis of starch, **J**-Growth at 65°C, **K**-Rod breadth 1μ or more, **L**-pH in VP broth <6.0, **M**-Hydrolysis of casein



**Fig. 1** Genetic map of T/A vector



**Fig. 2.** Gram staining of isolates from predigester (a) *B. silvestris* (b) *B. thuringenesis* (c) *B. firmus* (d) *B. licheniformis* (e) *B pumilus* (f) *B .cereus* and phase contrast micrographs showing spores of (g) *B. thuringenesis* (h) *B. megaterium* (i) *B. silvestris*



**Fig. 3.** Ziehl Neelsen staining technique showing (a) parasporal bodies attached to the spores in *B. thuringenesis* and (b) their absence in *B. cereus*