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# ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF FOUR CELLULOLYTIC ACTINOMYCETES AND THEIR CELLULASES

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## ABSTRACT

Four highly cellulolytic actinomycetous isolates namely $SG_1$, $SG_2$, $SG_3$ and $SS_1$ were isolated from soil samples and provisionally identified as *Streptomyces almquistii*, *S. caeruleus* , *S. hirsutus* and *S. endus*, respectively. All the isolates showed heavy growth and liquefaction at 50˚C and pH 6.5 in Winstead's medium having 1.2% of CMC. The isolates were allowed to grow in Winstead's medium having Asparagine as a nitrogen source with different carbon sources for the maximum production of cellulases. The extracellular protein of the culture supernatant ranged from 1.14 µg /ml ($SG_1$) to 879.39 µg /ml ($SG_3$). The reducing sugar level of the culture supernatant ranged from 0.76 µg /ml ($SG_2$) to 558.33 µg /ml ($SG_1$). The highest CMC-ase activity (1431.81U/ml) was found with the crude enzyme of the strain $SG_3$. The highest FP-ase activity (1087.11 U/ml) and Avicelase activity (1287.87U/ml) were found with the crude enzyme of $SS_1$. To detrermine the optimum nitrogen sources, the isolates were allowed to grow in Winstead's medium having saw dust for $SG_1$and $SG_3$, dry leaf for $SS_1$ and $SG_2$ as a carbon source with different nitrogen sources for the maximum production of cellulases. The extracellular protein of the culture supernatant ranged from 35.50 µg /ml ($SG_1$) to 328.62 µg /ml ($SS_1$) and the reducing sugar level of the culture supernatant ranged from 3.79 µg /ml ($SG_1$) to 114.39 µg /ml ($SG_3$). However the highest CMC-ase activity (1353.78 U/ml), and FP-ase activity (215.90 U/ml) were found with the crude enzyme of the strain $SG_2$ and Avicelase activity (356.06U/ml) was found with the crude enzyme of the isolate $SS_1$.

**Key words:** Streptomyces, Winstead's

## INTRODUCTION

Cellulose is a long-chain polysaccharide of β-glucose and the most abundant organic compound on earth. The primary cell wall of green plants is made primarily of cellulose and the secondary wall contains cellulose with variable amounts of lignin. Lignin and cellulose, considered together, are termed

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lignocellulose, which is the most common biopolymer on Earth. While humans cannot digest cellulose, many even-toed ungulates and termites can digest cellulose through a mutually beneficial symbiotic relationship with particular microorganisms that can break down the cellulose to usable form (Updegraff 1969, Crawford 1981, Ozturk *et al*. 2006,). Cellulolysis is the process of breaking down cellulose into smaller polysaccharides called cellodextrins or completely into glucose units which is a hydrolysis reaction. Because cellulose molecules bind strongly to each other, cellulolysis is relatively a difficult process compared to the break down of other polysaccharides (David *et al*. 2008)

Most mammals have only very limited ability to digest cellulose. Some ruminants like cows and sheep contain certain symbiotic anaerobic bacteria (like *Cellulomonas*) in the flora of the rumen which produce enzymes called cellulases that help the microorganism to break down cellulose and the breakdown products are then used by the bacteria for proliferation. The bacterial mass is later digested by the ruminant in its digestive system like stomach and small intestine (Tokuda and Watanabe 2007). Although cellulases are distributed throughout the biosphere, they are mostly found in fungi and other microbial sources. The actinomycetes are an important part of the microbial community in the soil environment, responsible for degradation and recycling of natural biopolymers, such as cellulose, lignin and chitin (Semedo *et al*. 2001) and also a source of a wide range of other types of bioactive compounds for biotechnological applications (Okami and Hotta 1988, Bull *et al*. 1992).

In this study, we describe the isolation and characterization of 4 cellulolytic actinomycetes to find out the optimum conditions for growth and enzymatic activities of the isolates.

## MATERIALS AND METHODS

### *Substrate Preparation*

Saw dust, rice bran, sugarcane baggage, coconut husk, dry leaf and CMC were used as substrates. The natural cellulosic substrates were pretreated by boiling in 0.5% NaOH for 1 hour following the Gray method (Gray *et al.*1978)

### *Microorganisms*

Four *Streptomyces* species ($SG_1$, $SG_2$, $SG_3$ and $SS_1$) were isolated from soil. After isolation the organisms were purified through repeated plating in Nutrient Agar medium. On the basis of morphological and cultural characteristics

the isolates $SG_1$, $SG_2$, $SG_3$ and $SS_1$were provisionally identified as *Streptomyces almquistii* (Duche 1934) *Streptomyces caeruleus*(Baldacci 1944). *Streptomyces hirsutus* (Ettlinger *et.al.*1958) and *Streptomyces endus* (Anderson and Gottlieb 1952) respectively.

*Biomass yield*

The filter paper containing biomass residue was dried in oven at $80^0$C for a constant weight and the amount of biomass was calculated.

*Optimization of cultural conditions*

An attempt was also made to determine the optimum culture conditions such as pH, temperature, carbon and nitrogen source requirements for their maximum growth and activities. The biomass yield, extracellular protein, reducing sugar level and cellulase production of the isolates was recorded.

*Medium pH*

To observe the effect of medium pH on enzyme production, selected medium pH of 4.5, 6.5, 7.0, 7.5 and 8.5 was inoculated with the isolates. The effects of medium pH on growth and liquefaction were recorded.

*Temperature*

To determine the optimum temperature for enzyme production the culture medium was incubated at 10˚, 27˚, 37˚, 45˚, 50˚ and 55˚C temperature at optimum pH and incubation period. The effects of temperature on growth and liquefaction were recorded.

*Carbon and nitrogen sources*

The production of cellulase under different carbon and nitrogen sources were studied in the liquid culture medium. Six carbon (CMC, Saw dust, Rice bran, Sugarcane baggage, Dry leaf and Coconut husk) and five nitrogen (Asparagine, Urea, Beef extract, Yeast extract and Peptone) sources were added to the medium and the effect of this carbon and nitrogen sources on the production of cellulase, extracellular protein, reducing sugar level and biomass yield were recorded.

*Enzyme assay*

For CMC-ase activity 2 ml of filtrate was added to 2 ml of 1% CMC and 1 ml of citrate phosphate buffer (pH 7.0), for FP-ase activity 2 ml of filtrate was

added to 1 ml of citrate phosphate buffer along with 50 mg Whatman No-1 filter paper strip (1x6 cm) and for Avicilase activity 2 ml of filtrate was added to 2 ml of 1% Avicel and 1 ml of citrate phosphate buffer in a test tube and incubated at $37^0$C for 2 hours in a water bath. The amount of reducing sugars released in CMC-ase, FP-ase and Avicilase assay after incubation was measured by Nelson's modification of Somogyi method (Somogyi 1944). Enzyme activity was expressed by the amount of glucose released in µg/ml of crude enzyme/ hour enzyme-substrate reaction at given conditions (Mahadevan and Sridhar 1982). Soluble protein in cultrate filtrate was estimated following the Lowry method (Lowry *et al.* 1951).

*Saccharification*

Saccharification (%) was calculated by applying the following equation:

$$\text{Saccharification \%} = \frac{\text{mg. of reducing sugar per ml}}{\text{mg. of substrate per ml}} \times 100$$

## RESULTS AND DISCUSSION

*Effects of Medium pH and Temperature*

At low pH (4.5), all the isolates showed low growth and liquefaction in Winstead's medium having 1.2% of CMC. At pH 8.5 all the isolates showed moderate growth and liquefaction except $SG_3$, which showed heavy growth at pH 8.5. All the isolates showed heavy growth and liquefaction at pH 6.5. At 50˚C all the isolates showed heavy growth and liquefaction. The isolates $SG_2$ and $SG_3$ also showed heavy growth and liquefaction at 45˚C. But at 10˚C, all the isolates were found to be unable to degrade the cellulose (CMC). (Table 1)

Heavy growth at pH 6.5 to 7.5 with different microorganisms was reported by many workers (Malek *et al* .1987, Shailendra *et al.*1991, Hossain *et al.*1999, Farhana *et al.* 2000). Heavy growth of actinomycetes at temperature 50˚C was reported by many workers (Cresswell *et al*. 1988, Jang and Chen 2003).The present observation is in concurrence with their reports.

Both pH and temperature have an effect on cellulose liquefaction. The higher liquefaction of cellulose due to enzyme activity at pH 6.5 to 7.5 was reported by many workers ( Malek *et al*.1987, Araujo and Ward 1990, Hachiro

and Kazuhiko 1991, Shailendra *et al.* 1991, Hossain *et al*. 1998, Hossain *et al.*1999).The higher liquefaction of cellulose due to enzyme activity at 50˚C was reported by Kaneko *et al*. 2005 and Lee *et al*. 2006. This observation also showed similarities with their reports.

TABLE 1: EFFECT OF pH AND TEMPERATURE ON THE GROWTH AND LIQUEFACTION OF THE SELECTED ISOLATES.

| Isolate | pH | | | | | Temperature (˚C) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Nos | 8.5 | 7.5 | 7.0 | 6.5 | 4.5 | 10 | 27 | 37 | 45 | 50 | 55 |
| $SG_1$ | ++ | ++ | ++ | + + + | + | _ | + | + | ++ | + + + | ++ |
| $SG_2$ | ++ | ++ | ++ | + + + | + | _ | + | + | + + + | + + + | ++ |
| $SG_3$ | +++ | ++ | ++ | + + + | + | _ | + | ++ | + + + | + + + | ++ |
| $SS_1$ | ++ | ++ | ++ | + + + | + | _ | + | ++ | ++ | + + + | ++ |

+,++. and +++ = Indicate low, moderate and heavy growth/ liquefaction respectively.

*Effects of Carbon sources*

The isolates were allowed to grow in Winstead's medium having Asparagine as a nitrogen source and 1.2% of CMC / Saw dust/ Rice bran/ Sugarcane baggage/ Coccunut husk/ Dry leaf as a carbon source for the determination of optimum carbon sources for maximum production of cellulase, reducing sugar level, extracellular protein, saccharificaton(%) and biomass (Table 2). The change of pH of the culture supernatant ranged from 7.5 to 8.4.

The extracellular protein of culture supernatant of the isolates $SG_1$, $SS_1$, $SG_2$ and $SG_3$ ranged from 1.14 µg /ml (sugarcane baggage) to 588.55 µg /ml (coccunut husk) , 17.17 µg /ml (dry leaf) to 677.86 µg /ml (CMC), 53.82 µg /ml (Saw dust) to 480.92 µg /ml ( Rice Bran) and 9.16 µg /ml (Dry leaf) to 879.39 µg /ml (CMC) respectively. and Reducing sugar level of the isolates $SG_1$, $SS_1$, $SG_2$ and $SG_3$ ranged from 7.57 µg /ml (coconut husk) to 558.33 µg /ml (saw dust), 18.94 µg /ml (saw dust) to 302.27 µg /ml (sugarcane baggage), 0.757 µg /ml (Sugarcane baggage) to 459.09 µg /ml (Dry leaf) and 4.54 µg /ml (Sugarcane baggage) to 365.91 µg /ml (Saw dust) respectively.

Highest saccharification percentage for the isolates $SG_1$, $SS_1$, $SG_2$ and $SG_3$ were found 4.65% (saw dust), 2.52% (sugarcane baggage), 3.82% (Dry leaf) and 3.05% (Saw dust) and highest biomass yield were found 411.67 mg/gm (rice bran), 348.33 mg/gm (sugarcane baggage), 266.67 mg/gm (Coconut husk) and 281.67 mg/gm (Dry leaf) respectively.

*Effect of Nitrogen sources*

The isolates were then allowed to grow in Winstead's medium having saw dust for $SG_1$and $SG_3$ and dry leaf for $SS_1$ and $SG_2$ as a carbon source and Asparagine/Urea/Beef extract/Yeast extract/Peptone as a nitrogen source for the determination of optimum nitrogen sources for maximum production of cellulase, reducing sugar level, extracellular protein, saccharification (%), biomass (Table 3). The change of pH of the culture supernatant ranged from 7.7 to 8.6.

Extracellular protein of culture supernatant of the isolates $SG_1$, $SS_1$, $SG_2$ and $SG_3$ ranged from 35.50 µg /ml (Asparagine) to 262.21 µg /ml (Beef extract), 92.75 µg /ml (Beef extract) to 328.62 µg /ml (Urea) , 49.24 µg /ml (Urea) to 211.83 µg /ml (Peptone) and 92.75 µg /ml (Asparagine) to 248.47 µg /ml (Peptone) respectively. Reducing sugar level of the isolates $SG_1$, $SS_1$, $SG_2$ and $SG_3$ ranged from 3.79 µg /ml ( Peptone) to 56.81 µg /ml(Urea), 18.18 µg /ml (Peptone) to 70.45 µg /ml (Asparagine), 5.30 µg /ml ( Peptone) to 28.03 µg /ml(Beef extract) and 7.57 µg /ml (Peptone) to 114.39 µg /ml (Urea) respectively.

Highest saccharification percentage of the isolates $SG_1$, $SS_1$, $SG_2$ and $SG_3$ were found 0.47% (Urea), 0.58% (Asparagine), 0.23% (Beef extract) and 0.95% (Urea) and highest biomass yield were found 236.67 mg/gm (Peptone), 331.67 mg/gm (Peptone), 351.67 mg/gm (Asparagine) and 228.33 mg/gm (Peptone) respectively.

TABLE 2: EXTRACELLULAR PROTEIN, REDUCING SUGAR LEVEL, BIOMASS YIELD AND SACCHARIFICATION (%) OF THE SELECTED ISOLATES ON DIFFERENT CARBON SOURCES.

| Sources of carbon | Isolate Nos | Final pH | Extra-cellular protein µg /ml | Reducing sugar µg/ml | Biomass yield mg/ml cellulose | Saccharification (%) |
|---|---|---|---|---|---|---|
| CMC | $SG_1$ | 8.2 | 429.39 | 412.87 | 258.33 | 3.44 |
| | $SS_1$ | 8.3 | 677.86 | 81.82 | 213.67 | 0.68 |
| | $SG_3$ | 8.3 | 879.39 | 92.42 | 256.67 | 0.77 |
| | $SG_2$ | 8.3 | 61.83 | 22.73 | 218.33 | 0.19 |
| Saw dust | $SG_1$ | 7.7 | 580.53 | 558.33 | 195.00 | 4.65 |
| | $SS_1$ | 7.5 | 396.18 | 18.94 | 161.67 | 0.16 |
| | $SG_3$ | 7.6 | 113.36 | 365.91 | 241.67 | 3.05 |
| | $SG_2$ | 7.7 | 53.82 | 31.82 | 205.00 | 0.27 |
| Rice bran | $SG_1$ | 7.7 | 9.16 | 550.33 | 411.67 | 4.60 |
| | $SS_1$ | 8.2 | 177.48 | 18.98 | 335.00 | 0.16 |
| | $SG_3$ | 8.0 | 652.67 | 360.91 | 243.33 | 3.05 |
| | $SG_2$ | 8.2 | 480.92 | 31.82 | 200.00 | 0.27 |
| Sugarcane baggage | $SG_1$ | 7.5 | 1.14 | 389.39 | 173.33 | 3.24 |
| | $SS_1$ | 8.2 | 96.47 | 302.27 | 348.33 | 2.52 |
| | $SG_3$ | 8.2 | 81.30 | 4.54 | 246.67 | 0.03 |
| | $SG_2$ | 8.1 | 75.57 | 0.76 | 240.00 | 0.01 |
| Dry leaf | $SG_1$ | 8.2 | 19.46 | 11.36 | 178.33 | 0.09 |
| | $SS_1$ | 8.2 | 17.17 | 287.12 | 220.00 | 2.39 |
| | $SG_3$ | 8.1 | 9.16 | 7.57 | 281.67 | 0.06 |
| | $SG_2$ | 8.1 | 444.27 | 459.09 | 236.67 | 3.82 |
| Coconut husk | $SG_1$ | 8.2 | 588.55 | 7.57 | 176.67 | 0.06 |
| | $SS_1$ | 8.1 | 253.05 | 23.48 | 193.33 | 0.19 |
| | $SG_3$ | 8.4 | 502.67 | 159.85 | 215.00 | 1.33 |
| | $SG_2$ | 8.3 | 195.80 | 26.51 | 266.67 | 0.22 |

TABLE 3: EXTRACELLULAR PROTEIN, REDUCING SUGAR LEVEL, BIOMASS YIELD AND SACCHARIFICATION (%) OF THE SELECTED ISOLATES ON DIFFERENT NITROGEN SOURCES.

| Sources of Nitrogen | Isolate Nos | Final pH | Extra-cellular protein µg /ml | Reducing sugar µg/ml | Biomass yield mg/ml cellulose | Saccharification (%) |
|---|---|---|---|---|---|---|
| Asparagine | $SG_1$ | 8.5 | 35.50 | 53.79 | 135.00 | 0.45 |
| | $SS_1$ | 8.4 | 123.66 | 70.45 | 166.67 | 0.58 |
| | $SG_3$ | 8.6 | 92.75 | 73.48 | 181.67 | 0.61 |
| | $SG_2$ | 8.4 | 84.73 | 24.24 | 351.67 | 0.20 |
| Urea | $SG_1$ | 8.4 | 91.60 | 56.81 | 171.67 | 0.47 |
| | $SS_1$ | 8.5 | 328.62 | 65.91 | 236.67 | 0.55 |
| | $SG_3$ | 7.9 | 217.56 | 114.39 | 191.67 | 0.95 |
| | $SG_2$ | 8.5 | 49.24 | 6.82 | 221.67 | 0.06 |
| Beef extract | $SG_1$ | 8.1 | 262.21 | 18.94 | 221.67 | 0.16 |
| | $SS_1$ | 7.7 | 92.75 | 34.09 | 316.67 | 0.28 |
| | $SG_3$ | 8.2 | 247.33 | 54.54 | 191.67 | 0.45 |
| | $SG_2$ | 8.3 | 156.87 | 28.03 | 133.33 | 0.23 |
| Yeast extract | $SG_1$ | 8.2 | 178.63 | 54.54 | 200.00 | 0.45 |
| | $SS_1$ | 8.5 | 201.53 | 41.67 | 311.67 | 0.35 |
| | $SG_3$ | 8.2 | 178.63 | 12.12 | 151.67 | 0.10 |
| | $SG_2$ | 8.2 | 85.88 | 12.12 | 338.33 | 0.10 |
| Peptone | $SG_1$ | 8.2 | 192.37 | 3.79 | 236.67 | 0.03 |
| | $SS_1$ | 8.3 | 199.24 | 18.18 | 331.67 | 0.15 |
| | $SG_3$ | 8.1 | 248.47 | 7.57 | 228.33 | 0.06 |
| | $SG_2$ | 8.0 | 211.83 | 5.30 | 333.33 | 0.04 |

*Enzyme activity*

The quantitative cellulase activity (CMC-ase, FP-ase & Avicelase) of crude enzymes produced by the isolates $SG_1$, $SS_1$, $SG_2$ and $SG_3$ grown in liquid Winstead's medium having 1.2% of CMC / saw dust/ rice bran/ sugarcane baggage/ coconut husk/ dry leaf (as a carbon source) were shown in Table 4.

Among the four isolates, the isolate $SG_3$ showed highest CMC-ase activity 1431.81 U/ml and $SG_2$ showed the lowest CMC-ase activity1.51 U/ml. The isolate $SS_1$ showed highest FP-ase activity 1087.11 U/ml and the lowest FP-ase activity 0.77 U/ml was recorded with crude enzyme of the strain $SG_1$.
The highest and lowest Avicelase activity was found 1287.87U/ml and 1.89 U/ml with the isolate $SS_1$ respectively.

Induction or repression of microbial cellulase enzyme production due to addition of different carbon sources to the cellulose medium was reported by many workers (Mandel and Reese 1957, Mandel *et al.*1962, Martin and Eberhart 1966, Mandel and Weber 1969, Nisizawa *et al.*1972. Breuli and Krushner 1976, Donald *et al.* 1995, Kashem 1998, Hossain *et al.*1999, Huq *et al.* 2002, Alam *et al.* 2004).The present observations are in concurrence with many of the above reports.

The quantitative cellulase activity (CMC-ase, FP-ase & Avicelase) of crude enzymes produced by the selected isolates while grown in liquid Winstead's medium having saw dust for $SG_1$and $SG_3$, dry leaf for $SS_1$ and $SG_2$ as a carbon source and Asparagine/ Urea/ Beef extract/ Yeast extract / Peptone as a nitrogen source are shown in the Table 5**.** The highest CMC-ase activity was 1353.78 U/ml and lowest was 7.57 U/ml with the crude enzyme of the isolate $SG_2$ was recorded.

The highest FP-ase activity 215.90 U/ml was recorded with the isolate $SG_2$ and lowest FP-ase activity 9.09U/ml with the crude enzyme of the strain $SG_1$.The highest Avicelase activity 356.06U/ml was observed with $SS_1$ and lowest Avicelase activity 11.36 U/ml was recorded with $SG_3$.

The induction or repression of microbial cellulase enzymes production due to addition of different nitrogen sources in the medium reported by some earlier workers (Shewale and Sadana 1968, Kashem 1998, Hossain *et al.*1999, Huq *et al.* 2002). In the present study both the induction and repression of cellulase production was recorded with different nitrogen sources.

Comparative study of enzyme production by the four actinomycetes indicated that CMC-ase activity was found higher compared to that of FP-ase and Avicelase activity, which is in accordance with the findings of many workers (Grag and Neelkanten 1982, Reddy 1984, Anwar and Zaman 1994, Rahman and Anwar 1996, Manchur and Anwar 1998, Shibli *et al.* 2001, Shibli *et al.* 2002, Alam *et al.* 2004, Alam *et al.* 2006).

The microbial biomass produced by the isolates indicated that the biomass yield and cellulase activity have no direct correlation. Similar observation have also been made by many other workers (Zaman, 1990, Mortuza 1993, Zakir 1994, Rahman and Anwar 1996, Manchur and Anwar 1998, Alam *et al.* 2004, Alam *et al.* 2006).

TABLE 4: RELATIVE CELLULOLYTIC ACTIVITIES OF CRUDE ENZYMES PRODUCED BY THE SELECTED ISOLATES ON DIFFERENT CARBON SOURCES.

| Sources of carbon | Isolate Nos | CMC-ase activity U/ml | FP-ase activity U/ml | Avicelase activity U/ml |
|---|---|---|---|---|
| CMC | $SG_1$ | 69.70 | 0.77* | 690.90 |
| | $SS_1$ | 829.54 | 1087.11** | 624.99 |
| | $SG_3$ | 1431.81** | 79.54 | 56.82 |
| | $SG_2$ | 83.33 | 571.96 | 785.60 |
| Saw dust | $SG_1$ | 45.45 | 266.66 | 35.98 |
| | $SS_1$ | 62.50 | 761.36 | 1.89* |
| | $SG_3$ | 37.89 | 286.36 | 1134.08 |
| | $SG_2$ | 577.27 | 5.30 | 32.20 |
| Rice bran | $SG_1$ | 41.67 | 11.29 | 278.03 |
| | $SS_1$ | 35.98 | 2.27 | 1270.44 |
| | $SG_3$ | 931.81 | 11.36 | 315.90 |
| | $SG_2$ | 1348.47 | 541.66 | 512.87 |
| Sugarcane baggage | $SG_1$ | 1418.17 | 225.75 | 3.79 |
| | $SS_1$ | 13.26 | 252.27 | 24.24 |
| | $SG_3$ | 872.72 | 313.18 | 925.75 |
| | $SG_2$ | 1.51* | 312.88 | 83.33 |
| Dry leaf | $SG_1$ | 280.30 | 9.09 | 16.67 |
| | $SS_1$ | 64.39 | 800.75 | 1287.87** |
| | $SG_3$ | 94.70 | 317.42 | 660.60 |
| | $SG_2$ | 46.97 | 410.60 | 18.94 |
| Coconut husk | $SG_1$ | 1249.99 | 3.03 | 384.09 |
| | $SS_1$ | 35.98 | 18.94 | 334.84 |
| | $SG_3$ | 319.69 | 12.12 | 473.48 |
| | $SG_2$ | 808.33 | 19.24 | 416.66 |

* and ** indicates minimum and maximum respectively

TABLE 5: RELATIVE CELLULOLYTIC ACTIVITIES OF CRUDE ENZYMES PRODUCED BY THE SELECTED ISOLATES WITH DIFFERENT NITROGEN SOURCES.

| Sources of $N_2$ | Isolate Nos | CMC-ase activity U/ml | FP-ase activity U/ml | Aviceles activity U/ml |
|---|---|---|---|---|
| Asparagine | $SG_1$ | 232.57 | 85.60 | 136.36 |
| | $SS_1$ | 217.40 | 9.09* | 356.06** |
| | $SG_3$ | 421.97 | 124.24 | 41.67 |
| | $SG_2$ | 1.89 | 25.76 | 45.45 |
| Urea | $SG_1$ | 300.75 | 28.03 | 183.33 |
| | $SS_1$ | 329.54 | 94.70 | 318.18 |
| | $SG_3$ | 53.03 | 195.45 | 11.36* |
| | $SG_2$ | 1353.78** | 11.36 | 106.06 |
| Beef extrtact | $SG_1$ | 175.76 | 22.73 | 75.76 |
| | $SS_1$ | 209.85 | 99.24 | 193.18 |
| | $SG_3$ | 190.91 | 64.39 | 96.21 |
| | $SG_2$ | 651.51 | 215.90** | 66.29 |
| Yeast extract | $SG_1$ | 194.69 | 34.85 | 68.18 |
| | $SS_1$ | 162.88 | 21.21 | 149.24 |
| | $SG_3$ | 31.82 | 25.00 | 22.73 |
| | $SG_2$ | 7.57* | 25.00 | 28.03 |
| Peptone | $SG_1$ | 380.33 | 121.97 | 143.94 |
| | $SS_1$ | 189.39 | 40.91 | 299.24 |
| | $SG_3$ | 149.24 | 46.21 | 136.36 |
| | $SG_2$ | 84.85 | 91.66 | 84.85 |

* and ** indicate minimum and maximum respectively

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