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RESEARCH ARTICLE

Studies on Enzymatic Characteristics of select Cultures for Preparation of Beneficial Microbial Consortium for Composting of Municipal Solid Waste

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ABSTRACT
The usual composting methods employed today involve use of native micro flora, less attention was given towards use of cultural amendments or microbial consortium for composting of the solid waste. This investigation is directed towards developing a microbial consortium for rapid and effective composting method. During the initial screening experiments for Cellulase, Lignin peroxidase, Xylanase, Phospahtase, Lipase, Protease, Urease and α- Amylase enzymes, 30 mesophilic bacterial cultures,17 thermophilic bacterial cultures, 18 mesophilic fungal cultures and 14 thermophilic fungal cultures were isolated. These cultures were quantitatively assessed for collective enzyme activities, using standard assay methods reported in literature.
Based on the qualitative analysis one representative culture each from mesophilic bacteria, thermophilic bacteria, mesophilic fungi and thermophilic fungi were selected with maximum activities for all the enzymes studied.
KEY WORDS: *Microbial consortium,municipal solid waste, Cellulase, Lignin peroxidase, Xylanase, Phospahtase,Lipase, Protease, Urease, α- Amylase.*

INTRODUCTION

Municipal Solid Waste Management (MSWM) is one of the critical environmental challenges of rapid urban development facing the developing countries including India. Solid waste arising from human domestic, social and industrial activities is increasing in quantity and variety as a result of growing population, rising standards of living and development of technology [1, 2]. Based on the nature the waste could be broadly classified as biodegradable and non-biodegradable.The biodegradable fraction of the MSW is very much detrimental to the surrounding environment due to its possibilities of yielding highly toxic species of chemicals.

Composting process has been developed for many reasons such as, higher decomposition rates and lower stabilization time, odorless conditions and producing higher temperatures resulted to higher safety from the point of view of pathogen and parasite destruction [3]. The composting process practiced today utilize the inbuilt or native flora for the purpose, very little efforts have been taken towards using any cultural amendments or consortia to carry out the composting. Some efforts with respect to use of microbial amendments in the composting process have been taken [4]. Although the present practice is satisfactory with respect to effective disposal of the solid waste, but it requires about 8 to 10 weeks for completion.

Hudson [5] described succession in aerobic process, noting that the composition of active microflora of the composting waste from predominantly mesophilic in the early stages of thermo genesis to one of predominantly thermophiles at peak of the healing cycle. He identified the mesophilic *Cladosporium herbarium, Aureobasidium sp., Alternaria sp.* and *Epicoccum purpurascens* at the beginning of composting process. Hamer [6] suggested that unlike microbially mediated production processes, microbially mediated environmental protection and restoration processes involve process cultures comprising multiple microbial consortia.

This investigation deals with the selection and isolation of potential beneficial microorganisms for carrying out a rapid and efficient composting of the MSW of Aurangabad City, and to develop a Beneficial Microbial Consortia (BMC) for carrying out faster composting of the municipal solid waste. The microorganisms were isolated from the black garden soils rich in organic contents using suitable enrichment and isolation techniques. The isolation was done at two temperature ranges 30°C and 55°C for isolation of the mesophilic and thermophilic cultures respectively. The enzyme studies were done for, Cellulase, Lignin peroxidase, Xylanase, Phospahtase, Lipase, Protease, Urease and α-Amylase. Separate selective media were used to detect individual enzyme. The cultures selected in the screening process were then individually subjected to quantitative analysis of various significant enzymes, using suitable assay techniques.

The cultures with the maximum activities for all the desired enzymes collectively were selected. The selected bacterial cultures were then identified at NCL Pune by 16s RNA analysis. The fungal cultures were identified by staining and morphological patterns.

MATERIAL AND METHODS

Enzymatic Assays for the selected isolates

Cellulase Assay

- CMC solution (0.5 ml) and 0.5 ml of culture supernatant and 0.5 ml of 0.05M phosphate buffer (pH 7.2) were incubated at 30°C for mesophiles and 60°C for thermophiles for 30 minutes.
- After incubation, 2ml of Dinitro Salisylic Acid (DNSA) reagent was added to the tubes and boiled for 15 minutes.
- The reaction mixture was cooled and optical density was measured at 540 nm and compared with standard graph of maltose [7].

Lignin Peroxidase assay

- H_2O_2 (0.1ml of 100µM), 0.1 ml of 32mM of Azure B and 0.8 ml of the culture supernatant were added in a test tube. The reaction was carried for 25 minutes. The absorbance was recorded at 651nm. The Lignin peroxidase activity was estimated from the standard graph.

Xylanase assay

- Culture filtrates (1 ml) was taken in test tubes; to this 1ml of 1% xylan solution and 1 ml of Citrate buffer (pH 5.5) were added. The mixture was incubated at 30°C for 30 minutes (Unshaken).
- The reaction was stopped by addition of 1 ml of Copper reagent (Solution C) and kept in boiling water for 10 minutes.The reaction mixture was cooled at 30°C and 1 ml of arsenomolybdate reagent (Solution D) was added and mixed thoroughly. To the mixture 15 ml of water was added and after proper mixing and centrifugation absorbance of the mixture was recorded at 500nm.
- The resulting sugar is estimated as xylose from the standard graph [8].

Phosphatase assay

- One ml of culture filtrate was incubated with 1 ml of 5mM *p*-nitrophenyl phosphate as the substrate, 1 ml of 0.5M Tris-HCl buffer (pH 5.6) and 1 ml mineral solution.
- After incubation at 30°C for 30 minutes at respective temperatures, the concentration of *p*-nitrophenol was determined at 400 nm on a Spectrophotometer.
- Phosphatase producers are defined as those which liberate more than 0.04 µM of *p*-nitrophenol per hour per milliliter of bacterial suspension [9].

Lipase Assay

- Olive oil emulsion (5 ml) was mixed with 2 ml of 0.1M Phosphate buffer of pH 7.0 and 1 ml of culture supernatant. The reaction mixture was incubated at 30°C for 30 minutes with shaking.
- The reaction was terminated by addition of 15 ml of ethanol: acetone [1:1 (v/v)] mixture.
- The fatty acids released were titrated with 0.05M Sodium hydroxide solution.
- One unit of lipase activity was defined as the amount of enzyme required to release one µmol of oleic acid in one minute per ml under the specified conditions, [10].

Protease Assay

- The 145µl of casein solution and 5 µl of the culture supernatants were mixed in wells of microtitre plates. The plate was incubated at 30°C for two hours. The turbidity before and after incubation in the microtitre wells was measured by microplate reader at a wave length of 595nm.

- The decrease in turbidity in the wells was used to evaluate casein digestion. The protease activity that could decrease the absorbance of 0.278 after two hours at 30°C was defined as one protease unit [11].

Urease Assay

- Culture filtrate (0.1 ml) was taken in microtubes, to this 0.5 ml of 50mM Urea and 0.5 ml of 100mM of Potassium phosphate buffer (pH 8.0) were taken.
- The tubes were incubated at 30°C for 30 minutes. The reaction was stopped by transferring 0.5 ml of the mixture to tubes containing 5 ml of phenol sodium nitroprusside solution.
- Alkaline hypochlorite (0.5 ml) was added to this tube and incubated at 30°C for 30 minutes.
- The optical density of color complex was measured at 630 nm, and compared with standard curve of $(NH4)_2SO_4$. [12].

Amylase Assay

The substrate used is starch solution rest procedure is same as for Cellulase assay.

Identification:

Identification of bacterial cultures:

The bacterial cultures were identified by 16S RNA analysis at National Chemical Laboratory, Pune.

Identification of fungal cultures:

The fungal cultures isolated were identified on the basis of their morphological features by staining with lactophenol cotton blue. A wet mount of fungal growth with lactophenol cotton blue was prepared on the slide and was observed under microscope at 40X. The morphological features were compared and the identification of the fungal isolate was confirmed.

RESULTS AND DISCUSSION

Screening for the beneficial microorganisms

A total of 30 mesophilic bacteria, 17 thermophilic bacteria, 18 mesophilic fungi and 14 thermophilic fungi were screened with collective enzyme activities for above listed enzymes.

Cellulase Assay

All the 30 mesophilic bacterial isolates were subjected to quantitative estimation of the Cellulase activity. Among the mesophilic bacterial isolates, isolate 2 has shown maximum activity with 15.9 units/ml. Isolates 5, 13, 14, 17, 18, 27, 29 and 30 were found negative for this enzyme. The overall cellulose activity of mesophilic bacterial isolates ranged from 0.4 units/ml to15.9 units/ml. Among thermophilic bacterial isolates (17), the cellulose activity ranged from 1 unit/ml to 15.1 units/ml. Isolate 12 has shown maximum activity with 15.1 units/ml. Isolate 9 and 10 were failed to produce this enzyme.

Among 18 mesophilic fungal isolates, isolate 9 has shown maximum activity with 15.2 unit/ml. In isolate 3 and 11 Cellulase activity was absent. The cellulase activity in all the cultures selected, ranged from 2.1 units/ml to 15.2 units/ml. Among 14 thermophilic fungal isolates, the activity ranged from 1.1 units/ml to 14.2 units/ml. The maximum cellulase activity was found in isolate 11 with 14.2 units/ml. Cellulase was not produced by isolate 9.

Lignin Peroxidase Assay

All the isolates were subjected to quantitative estimation of the Lignin Peroxidase activity. Among the mesophilic bacterial isolates, isolate, 13, 14, 18 and 22 have been found negative for Lignin Peroxidase. The overall activity for all mesophilic bacterial isolates ranged from 0.21 units/ml to 4.6 units/ml. Maximum activity was observed was 4.6 units/ml for isolate 19. Among 17 thermophilic bacterial isolates the activity ranged from 0.7units/ml to 4.8 units/ml. Isolate 7 has shown maximum activity with 4.8 units/ml. Isolate 9, 10 and13 were found to be deficient in this enzyme.

Among 18 mesophilic fungal isolates, isolate 2 has shown maximum activity with 4.6 unit/ml. In isolate 3 and 11 Cellulase was absent. The Lignin Peroxidase activity in all the cultures ranged from 0.9 units/ml to 4.6 units/ml. Among 14 thermophilic fungal isolates, the activity ranged from 1.1 units/ml to 5.2 units/ml. The maximum Lignin Peroxidase activity was found in isolate 11 with 5.2 units/ml. Lignin Peroxidase was absent in isolate 9 and 10.

Xylanase Assay

Xylanase activity was quantitatively estimated in all the isolates. Among the mesophilic bacterial isolates, isolates, 17, 18, 29 and 30 have been found negative for Xylanase. The overall activity for all

mesophilic bacterial isolates ranged from 0.29 units/ml to 4.9 units/ml. Maximum Xylanase activity was observed was 4.9 units/ml for isolate 2. Among 17 thermophilic bacterial isolates, the Xylanase activity ranged from 0.9 units/ml to 5.1 units/ml. Isolate 12 has shown maximum Xylanase activity with and 5.1 units/ml. Isolate 9 and13 were found to be deficient in Xylanase activity.

Among 18 mesophilic fungal isolates, isolate 2 has shown maximum activity with 5.1 unit/ml. In isolate 3 and 11 Xylanase was absent. The Xylanase activity in all the selected cultures ranged from 0.7 units/ml to 5.1 units/ml. Xylanase activity of al the 14 thermophilic fungal isolates, ranged from 0.9 units/ml to 5.8 units/ml. The maximum Xylanase activity was found for isolate 11 with 5.2 units/ml.

Acid Phosphatase Assay

Assay for Acid Phosphatase was carried out for all the isolates. The Acid Phosphatase activity of all the mesophilic bacterial isolates ranged from 0.22 units/ml to 7.1 units/ml. Maximum Acid phophatase activity was observed was 7.1 units/ml for isolate 2. Acid Phophatase activity of all the 17 thermophilic bacterial isolates ranged from 0.8 units/ml to 7.2 units/ml. Isolate 7 has shown maximum activity with 7.2 units/ml.

Among 18 mesophilic fungal isolates, isolate 9 has shown maximum acid Phosphatase activity with 7.3 unit/ml. The enzyme activity of all the cultures ranged from 0.8 units/ml to 7.3 units/ml. Among 14 thermophilic fungal isolates, the Acid Phosphatase activity ranged from 0.8 units/ml to 7.2 units/ml. The maximum activity was found in isolate 5 with 7.2 units/ml.

Lipase Assay

Assay for Lipase was done for all the isolates. The overall Lipase activity of mesophilic bacterial isolates ranged from 0.1units/ml to 32.2 units/ml. Maximum activity (32.2 units/ml) was observed for isolate 2. The Lipase activity of 17 thermophilic bacterial isolates, ranged from 1.2units/ml to 30.9 units/ml. Isolate 7 has shown maximum Lipase activity with and 30.9units/ml.

Among 18 mesophilic fungal isolates, isolate 9 has shown maximum Lipase activity with 27.6 unit/ml. The Lipase activity of all the cultures ranged from 1.1 units/ml to 27.6 units/ml. Among 14 thermophilic fungal isolates tested, the Lipase activity ranged from 1.1 units/ml to 30.1 units/ml. The maximum Lipase activity was found in isolate 5 with 30.1 units/ml.

Protease Assay

Assay for Protease was done for all the isolates. The Protease activity for all mesophilic bacterial isolates ranged from 0.66 units/ml to 13.2 units/ml. Maximum Protease activity was observed was 13.2 units/ml for isolate 2. Among 17 thermophilic bacterial isolates, the Protease activity ranged from 1.5 units/ml to 12.1 units/ml. Isolate 12, tested for Protease activity has shown maximum activity with 12.1 units/ml.

Among 18 mesophilic fungal isolates tested for Protease activity, isolate 9 showed maximum activity with 11.9 unit/ml. The Protease activity of all the cultures ranged from 0.9units/ml to 11.9 units/ml. For all the 14 thermophilic fungal isolates, the Protease activity ranged from 2.1units/ml to 11.7 units/ml. The maximum activity was found in isolate 5 with 11.7 units/ml.

Urease Assay

Assay for Urease was done for all the isolates. The Urease activity for all the mesophilic bacterial isolates ranged from 5.3 units/ml to 62.1 units/ml. Maximum Urease activity was observed was 62.1 units/ml for isolate 2. The Urease activity of all the 17 thermophilic bacterial isolates, ranged from 0.8 units/ml to 60.3 units/ml. Isolate 12 has shown maximum Urease activity with 60.3 units/ml.

Among 18 mesophilic fungal isolates, isolate 9 has shown maximum Urease activity with 54.2 unit/ml. The Urease activity in all the cultures ranged from 0.8 units/ml to 54.2 units/ml. Among 14 thermophilic fungal isolates, the activity ranged from 2.1 units/ml to 52.1 units/ml. The maximum activity was found in isolate 5 with 52.1 units/ml.

α- Amylase Assay

All the isolates were subjected to quantitative estimation of the α- Amylase activity. The overall activity for all mesophilic bacterial isolates ranged from 0.44 units/ml to 27.1 units/ml. Maximum activity was observed was 27.1 units/ml for isolate 2. The α- Amylase activity for all the 17 thermophilic bacterial isolates, ranged from 3.5 units/ml to 23.4 units/ml. Isolate 12 has shown maximum α- Amylase activity with 23.4 units/ml.

Among 18 mesophilic fungal isolates, isolate 2 has shown maximum activity with 26.1 unit/ml. The α- Amylase activity for all the cultures selected ranged from 2.9 units/ml to 26.1 units/ml. The α-

Amylase activity of thermophilic fungal isolates, ranged from 7.2 units/ml to 28.7 units/ml. The maximum α- Amylase activity was found in isolate 11 with 28.7 units/ml.
Based on the results obtained, among mesophilic bacterial isolates, isolate **2** has showed maximum collective activity for the selected seven enzymes. For thermophilic bacteria, isolate **12** has shown maximum collective activity for six enzymes. For the mesophilic fungal cultures, isolate **9** has shown maximum collective activities for five enzymes and for the thermophilic fungal cultures, isolate **5** has given maximum collective activities for five enzymes.
Hence mesophilic bacterial isolate **2**, thermophilic bacterial isolate **12**, mesophilic fugal isolate **9** and thermophilic fugal isolate **5** were selected for the development of the BMC. The results of quantitative enzymatic assays for mesophilic bacteria, thermophilic bacteria, mesophilic fungi and thermophilic fungi are tabulated in Tables 1 to 4.

Identification of the selected isolates for development of Beneficial Microbial Consortium (BMC)

The finally selected four cultures for development of BMC for conversion of organic garbage,were subjected to identification. The bacterial cultures were identified on the basis of 16S rRNA analysis. This identification was carried at NCL, Pune since facilities were not available the laboratory. The 16S rRNA analysis has revealed the two isolate to be *Bacillus subtilis* (mesophilic isolate 2) and *Bacillus sphaericus* (thermophilic isolate 12).
The fungal cultures were identified by standard staining methods, by staining with lactophenol cotton blue and comparison of the morphology with standards. On the basis of the morphological characterization the fungal isolates were identified as to be *Thermomyces lanuginosus* (thermophilic fungal isolate 5) and *Aspergilus niger* (mesophilic fungal isolate 9).

Table 1: Enzyme activities for screening of mesophilic bacterial isolates

Culture isolate	Enzyme Activities (Units/ml)							
	Cellulase	Lignin Peroxidase	Xylanase	Acid Phosphatase	Lipase	Protease	Urease	α-Amylase
1	5	1	2.1	0.67	9.21	3.52	21.3	7.9
2	**15.9**	**4.3**	**4.9**	**7.1**	**32.8**	**13.2**	**62.1**	**27.1**
3	13.2	3.1	2.1	2.3	12.1	4.9	43.1	14.3
4	2.5	2.4	3.5	4.3	19.4	6.5	44.4	20.3
5	0	1	1	1.7	6.7	2.4	31.2	12.9
6	1.9	0.8	1.3	3.1	15.2	4.8	33.4	9.7
7	12.5	2.8	0.7	1.2	16.3	3.1	29.4	2.3
8	13.7	3.4	4.2	1.1	16.2	3.21	38.1	1.5
9	3.7	0.8	1.4	0.72	3.2	2.7	0	1.1
10	4.2	0.59	1.6	0.92	1.1	1.9	0	2.1
11	2.1	0.92	0.78	0.82	2.2	0.7	11.3	3.4
12	9.6	3.2	2.4	3.8	12.2	9.4	28.7	8.5
13	0	0	0.9	1.3	8.3	15	21	4.2
14	0	0	0.3	0.22	5.4	2.9	0	1.8
15	4.8	1.9	2.7	2.5	6.4	4.1	9.3	5.2
16	3.5	1.6	1.9	0.43	0.97	1.9	0	1.1
17	0	1.3	0	2.6	11.1	8.1	9.2	0.44

Table 1 continued

Culture isolate	Enzyme Activities (Units/ml)							
	Cellulase	Lignin Peroxidase	Xylanase	Acid Phosphatase	Lipase	Protease	Urease	α-Amylase
18	0	0	0	1.1	2.6	3.1	6.2	1

19	14.2	4.6	4.8	6.9	31.1	12.8	57.2	21.3
20	1.4	1.3	0.31	0.42	0.1	1.3	0	0.93
21	1.1	3.2	0.29	1.1	0.42	1.92	0	0.86
22	0.9	0	1.4	2.2	0.39	3.61	11.2	0.53
23	3.2	0.3	2.1	1.6	0.26	2.18	16.3	1.89
24	5.4	0.21	3.1	1.4	0.88	2.78	12.2	1.95
25	10	2	2.4	2.3	1.21	0.81	10.5	2.43
26	0.7	1	1.8	2.5	1.93	3.28	9.3	4.58
27	0	1.2	1.1	0.43	2.88	3.84	8.4	3.41
28	0.4	3.2	0.3	1.8	3.21	1.65	7.1	9.33
29	0	1.8	0	1.2	9.78	0.66	6.9	5.29
30	0	0.32	0	1.8	4.56	1.77	5.3	6.16

Table 2: Enzyme activities for screening of thermophilic bacterial isolates.

Culture isolate	Enzyme Activities (Units/ml)							
	Cellulase	Lignin Peroxidase	Xylanase	Acid Phosphatase	Lipase	Protease	Urease	α-Amylase
1	3.2	0.7	1.7	1.2	2.1	2.1	15.1	7.2
2	3.1	0.9	2.8	3.2	1.2	3.1	10.9	8
3	2.3	0.8	2.5	1.8	6.7	8	15.1	9.1
4	5.4	3.2	2.9	4.1	14.1	5.4	16.9	9.2
5	1	0.8	2.1	2.2	4.3	5.3	19.8	17.2
6	8.2	1.1	3.1	2.3	13.2	6.1	9.2	7.8
7	13.9	4.8	4.3	6.7	30.9	11.3	55.2	22.1
8	9.2	3.1	2.4	0.8	10.2	1.8	15.3	4.3
9	0	0	0	1.1	2.1	3.1	1.3	4.3
10	0	0	0.9	2.3	2.4	3.2	1.5	4.5
11	1.5	1.1	0.9	4.1	3.4	1.5	16.2	6.4
12	**15.1**	**4.3**	**5.1**	**7.2**	**29.7**	**12.1**	**60.3**	**23.4**
13	1.1	0	0	2.4	6.4	8.2	10.9	5.9
14	2.3	0.8	1.1	2.5	3.1	4.2	0.8	3.5
15	8.1	2.3	1.5	3.2	21.9	6.8	31.9	8.6
16	4.6	0.9	1.7	2.7	4.2	2.3	0.8	3.5
17	2.3	1.7	1.4	3.5	12.4	7.4	33.2	14.3

Table 3: Enzyme activities for screening of mesophilic fungal isolates

Culture isolate	Enzyme Activities (Units/ml)							
	Cellulase	Lignin Peroxidase	Xylanase	Acid Phosphatase	Lipase	Protease	Urease	α-Amylase
1	2.9	1.5	2.4	2.8	3.5	4.6	17.2	8.5
2	14.9	4.6	5.1	6.8	27.2	10.2	49.2	26.1
3	0	0	0	2.1	1.8	2.1	1.9	5.4
4	6.8	3.2	3.6	5.4	12.1	5.7	12.8	6.5
5	2.3	1.7	1.6	1.6	3.2	3.1	9.8	18.5
6	6.3	2.3	1.9	4.2	11.8	5.1	7.3	12.3
7	8.2	3.2	2.6	3.7	13.9	6.5	21.9	15.6

8	7.5	2.9	3.4	3.1	6.8	3.7	10.2	8.7
9	**15.2**	**4.5**	**4.8**	**7.3**	**27.6**	**11.9**	**54.2**	**25.3**
10	3.1	1.8	2.3	1.8	1.9	8.2	30.1	18.9
11	0	0	0	3.2	2.1	2.2	19.2	8.9
12	4.1	3.2	3.1	6.2	20.9	10.1	38.2	22.1
13	3.1	2.4	1.9	4.2	3.8	5.4	9.2	18.7
14	2.1	0.9	0.7	1.2	1.1	0.9	0.8	2.9
15	2.9	1.8	0.9	0.8	7.1	2.7	8.9	7.7
16	2.6	1.1	1.4	3.1	2.5	2.9	1.9	4.9
17	2.3	1.7	1.4	3.5	4.8	6.1	7.9	5.1
18	5.4	1.9	2.8	3.3	12.2	1.1	10.4	7.2

Table 4: Enzyme activities for screening of thermophilic fungal isolates

Culture isolate	Enzyme activities (Units/ml)							
	Cellulase	Lignin Peroxidase	Xylanase	Acid Phosphatase	Lipase	Protease	Urease	α-Amylase
1	1.3	1.1	2.8	3.1	4.1	2.1	10.3	7.5
2	2.9	1.8	3.2	2.1	21.7	8.7	30.1	18.9
3	2.6	2.1	1.7	2.8	7.7	4.8	18.7	22
4	3.9	2.1	1.9	3.2	11.1	4.3	9.4	17.3
5	**13.2**	**4.9**	**5.8**	**7.2**	**30.1**	**11.7**	**52.1**	**27.4**
6	3.2	3.1	1.7	3	6.5	4.1	3.1	9.1
7	2.1	2.5	1.6	2.1	8.3	3.1	10.8	16.3
8	1.1	1.8	2.2	2.5	3.2	2.8	7.2	11.2
9	0	0	1.1	1.2	12.4	8.2	9.6	9.1
10	2.3	0	0.9	1.9	2.1	4.2	18.2	7.2
11	14.2	5.2	5.4	6.4	27.3	10.3	48.7	28.7
12	9.7	2.1	4.2	0.8	1.1	7.1	14.3	18.2
13	4.3	1.9	2.9	5.1	9.4	3.1	5.9	14.9
14	4.2	3.1	1.3	3.6	10.2	4.1	2.1	10.2

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