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Ethanol production from Egyptian sugar cane molasses by six yeast strains using batch fermentation

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Abstract: Six high ethanol producer yeast strains (two strains of *Kluyveromyces marixianus* and four of *Saccharomyces cerevisiae*) were utilized to produce ethanol from treated and non-treated Egyptian sugar cane molasses with gravity (10, 15, 20, 30 & 33.3% sugar). The treated molasses was obtained by heating diluted molasses up to 90ºC and adjusting its pH to 4.5. All yeast strains used produced higher ethanol yield from non-treated molasses with 10% sugar than that obtained from the treated one with the same sugar concentration. On the other hand, treated molasses yielded better ethanol concentration than that gained from non-treated molasses with 15 – 25% sugar. Maximum ethanol production (125.89% g/l) was noticed with fermentation efficiency of 99.97% using *S. cerevisiae* EC1118 strain on 25% sugar treated molasses at 35ºC. The same strain gave low levels of ethanol when the sugar concentration of the treated molasses was either 30 or 33.3% at both fermentation temperatures used (35º and 40ºC). The kinetic parameters and productivity were calculated and discussed for all treatments.

Key words: batch fermentation, ethanol, sugar cane molasses, yeast.

Introduction

Over the last two decades, natural energy resources such as petroleum and coal have been consumed at high rates. Therefore, the heavy reliance of the modern economy on these resources is bound to end. Also, due to their negative environmental impact in addition to the growing pressure of society as well as to the fact that these resources might eventually run out, alternative resources as ethanol have become badly needed. Bioethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels (Cardona and Sanchez, 2007). Some biological processes have rendered possible routes for producing ethanol in large volumes using cheap substrates (Gunasekaran and Raj, 1999).

Ethanol can be produced by fermentation of sugars from waste plant materials. Whatever substrate is chosen, the attention must be paid to the overall economics and energy consumption (Demirbas, 2006). The economic evaluation of different materials for ethanol production was thoroughly studied previously (Meo, 1984; Maiorella *et al.*, 1984; Greg and Saddler, 1995). Molasses (black syrup) is inedible for human, but it is primarily used as an animal feed additive and appeared to be a perfect feedstock for and substrate of production of alcohol, compressed yeast, citric acid as well as other organic acids and some therapeutic compounds (Jocques *et al.*, 2003). Molasses is produced in Egypt and other tropical countries and because of its low cost, it is an important economic source to produce many products by fermentation. Most of sugars in molasses are present in a readily fermentable form. The presence of these compounds in molasses may favour yeast growth and enable it for high ethanol levels of production.

Efficient ethanol production requires a rapid fermentation process leading to high ethanol concentrations. Therefore the candidate yeast strain must have a high growth rate and a good specific ethanol production rate at high osmotic tensions.

This study was designed to assess the capacity of six selected high-ethanol-producing yeast strains to produce ethanol from non-treated Egyptian sugar cane molasses with normal gravity (10, 15, 20 & 25% sugar) comparable with treated one with normal and high gravity (10, 15, 20, 25, 30 & 33.3% sugar) using batch fermentation technique.

Materials and Methods

Yeast Strains and Inoculum Preparation

Six yeast strains were used throughout this study. They comprised: two strains of *Kluyveromyces marixianus* (GU133331 & GU133329) which were previously recorded in our laboratory (Ali 2010) as high ethanol producers. Three strains of *Saccharomyces cerevisiae* (EC1118, CY3079 & GHM) which are used in brewing industry in Germany. The fouth strain was commercial compressed baker's yeast (*S. cerevisiae*) which was bought from a local grocery store. The inoculum was prepared by transferring one loopfull of 48 hours culture grown on a slant of YMPGA medium of the following composition: 3g yeast extract, 3g malt extract, 5g peptone, 10g glucose and 20 g agar, per one liter of water

(Wickerham, 1951) and dispensed in 50 ml sterilized YMPG broth aliquots in 250 ml Erlenmeyer flask. After incubation on a rotary shaker (150 rpm) at 30ºC for 48 hours, the inoculum was transferred at the rate of 10% (v/v) to the fermentation medium. The initial concentration of inoculum was maintained at 2×10^7 cells/ml in every case. Three replicates were used for each treatment.

Molasses

Egyptian sugar cane molasses used in this study was kindly provided by Naga–Hammady Co. Ltd. (Egypt). According to El-Samman (2010), the chemical constituents of Naga-Hammady molasses are: total sugar, 50.86%; ash, 11.21% and inorganic salts as P_2O_3, 0.17%; SO_2, 0.27%; Na_2O, 0.30% and K_2O, 3.69%, in addition to some nitrogenous materials such as proteins, amino acids and vitamins. Treated molasses was prepared by heating the diluted molasses at 90°C for 30 min using water bath after adjusting their pH to 4.5 using HCl (1N), while non-treated molasses was prepared by only diluting molasses to the required sugar concentration.

Ethanol Production

The six yeast strains were tested for their ability to produce ethanol from sugar cane treated and non-treated molasses with the following sugar concentrations: 10, 15, 20, 25, 30 & 33.3%. The fermentation process was conducted at 35º or 40ºC in 100 ml glass bottles containing 45 ml of treated or untreated sugar cane molasses and 10% (v/v) inoculum of tested yeast strain. The cultures were incubated on a rotary shaker (150 rpm) for 5 hours under aerobic conditions. Fermentation bottles were tightly sealed using parafilm and the fermentation process was completed under anaerobic conditions, as the CO_2 evolved during fermentation expels all the air from these bottles. Ethanol (E), total initial sugar (TIS), total residual sugar (TRS), dry biomass (B) and final pH (F_{pH}) values were measured.

Analytical Methods

Produced ethanol was estimated by bichromate method as described by Zohri and Mostafa (2000), while the volumetric ethanol productivity (V. E. P. g/l/h) and ethanol yield from theoretical value (YE of TH) were calculated according to Limtong *et al.* (2007). Fermentation efficiency (F. eff %) is expressed as g sugar utilized/100 g initial sugar according to Roukas (1996). Other parameters such as dry biomass concentration over the consumed sugar [YB/CS (g/g)], dry biomass concentration over the initial sugar [YB/IS (g/g)], ethanol concentration over the consumed sugar [YE/CS (g/g)] and ethanol concentration over the initial sugar [YE/IS (g/g)] as fermentation kinetics were calculated according to Siqueira *et al.* (2008). The dry biomass was determined by drying the yeast biomass for 24 hours at 85ºC and then weighed (Chanda and Chakrabarti, 1996). Total initial and residual sugars (TIS and TRS) were determined using the 3, 5- dinitrosalicylic acid (DNS) method (Miller, 1959) after neutralization with 1 N NaOH. Total consumed sugar (TCS) was calculated by subtracting TRS from TIS. PH value was measured by Microprocessor pH-mv meter 526.

Results and Discussion

In the present study, the fermentation performs of treated and non-treated molasses with 10% sugar concentration (Table 1) proved that all yeast strains used were able to produce ethanol. Ethanol concentrations ranged from 26.77 to 40.82 g/l (equivalent to 52.38% to 79.88% of the theoretical value) using treated 10% sugar molasses. On the other hand, non-treated 10% sugar molasses gave better yield of ethanol levels ranging from 38.86 to 48.61 g/l (76.05% to 95.12% of the theoretical value). It is worthy to mention that the use of the non-treated molasses saves the costs in addition to produce high levels of ethanol. *Saccharomyces cerevisiae* EC1118 was the best yeast strain to produce ethanol from the treated and non-treated cane molasses. Doelle *et al*. (1991) reported that 58.6 g/l of ethanol was obtained, as the highest level, by *Zymomonas mobilis* grown on cane molasses containing 10.9% sugar.

At 15 and 20% sugar, all yeast strains produced better yield of ethanol from treated molasses (44.55 – 89.02 g/l) than those produced when non-treated molasses was used (41.33 - 76.89 g/l) (Tables 2 & 3). Treated molasses yielded the highest ethanol level (125.89 g/l) at 25% sugar and *S. cerevisiae* EC1118 was the most potent strain used (Table 4). In this respect, Al-Talibi *et al.* (1975) found that the ethanol yield by *S. cerevisiae* using molasses with 20% sugar solution reached 97.65% of the theoretical value. The results of Roukas (1996) showed that the maximum ethanol level (43.5 g/l) was produced from non-sterilized beet molasses with 15% sugar by *S. cerevisiae* in batch culture. Cazetta *et al.* (2007) recorded that under the best conditions for ethanol production, *Z. mobilis* ATCC29191 formed 55.8 g/l ethanol from cane molasses with 20% sugar. Siqueira *et al*. (2008) registered 30.5 g/l of ethanol formed by *S. cerevisiae* LPB – JP grown on soybean molasses with 12.17% total sugars. Ali (2010) noticed that *Kluyveromyces marixianus* ZMS3-GU133329 produced 49.30 g/l ethanol when grown on synthetic medium containing 12.5% sugar concentration at 35°C. Zohri *et al* (2013) examined the suitability of beet molasses with 20% sugar for ethanol production by *S. cerevisiae* and found that the ethanol yield reached 91% of the theoretical value. Also, these results clearly showed that the

highest ethanol concentration attained using treated molasses (125.89 g/l) was greater than that formed from non-treated one (91.77 g/l) under the same experimental conditions by about 27.1%. These results are in harmony with those recorded by Kaseno and Kokugar (1997) who found that ethanol formed by *S. cerevisiae* from treated molasses (by filtration method) increased by about 18.1% over those formed from non-treated molasses under the same fermentation conditions.

The present results of the kinetics during ethanol production from 25% sugar cane molasses using the six yeast strains revealed that the ethanol productivities during the 120 hours of fermentation ranged from 0.65 to 1.09 and 0.34 to o.76 g/l/h using treated and non-treated 25% sugar molasses respectively (Table 4). Ethanol yield over either consumed sugar ($Y_{E/CS}$) or initial sugar ($Y_{E/IS}$) was nearly equal and ranged from 0.31 to 0.50 and 0.16 to 0.37 g/g, in both cases of treated and non-treated molasses respectively. These results are almost similar to those mentioned by Cazetta *et al* (2007) who reported that ethanol productivity during the fermentation of cane molasses with 25% sugar at 35^{o}C was 0.94 g/l/h and the $Y_{E/IS}$ was 0.40 g/g by *Z. mobilis*. Siqueira *et al.* (2008) determined the kinetics of bioethanol production from soybean molasses by *S. cerevisiae* at a laboratory scale and found that the ethanol yield ($Y_{E/CS}$) was 45.4% from consumed substrate representing 88.8% of the theoretical maximum.

The fermentation efficiencies of the six yeast strains using the treated and non-treated 25% sugar molasses ranged from 95.29% to 99.67% and 94.18 to 98.35% respectively (Table 4). Also, low levels of total residual sugar were recorded in treated molasses (1.18 – 0.08%) compared to those recorded in non-treated molasses (1.46 – 0.41%). These results agreed with those reported by Kaseno and Kokugan (1997). They examined the effect of molasses pretreated by microfiltration on ethanol production by *S. cerevisiae* and found that residual sugar in fermentation of pretreated molasses was reduced by about 42% as compared with non-pretreated one. They also reported that 90.2% of sugar in pretreated molasses was metabolized by yeast cells compared to 83.1% in non-pretreated molasses under the same experimental conditions.

When treated molasses with 30 and 33.3% sugar were used, the fermentation efficiency of *S. cerevisiae* at 35ºC increased to reach 99.54 and the total residual sugar decreased to 0.14%, yet the maximal ethanol yield decreased to 94.30 and 97.97 g/l respectively (Table 5). The final pHs after 72 hours of fermentation at 35^{o}C were slightly decreased to 4.30 and 4.15 from initial pH 4.5 in case of 30 and 33.3% sugar molasses respectively.

When the fermentation temperature was raised to 40^{o}C, maximum ethanol yield by *S. cerevisiae* EC1118 from molasses with 30 and 33.3% sugar reached 81.08 and 87.22 g/l (equivalent to 52.89% and 51.23% of the theoretical values) after 48 and 96 hours of fermentation respectively (Table 6). In a recent study, Lin *et al.* (2012) reported that the high substrate concentration (30%) may prevent the ethanol fermentation process and one of the reasons may be the accumulation of high ethanol concentration and by-products that make the pH change. Also, they recorded the formation of acetic acid was increased when the pH was below 4.0 and the ethanol could be utilized by the yeast as the carbon source after other nutrients became depleted. So, our results could also be explained in the light of the above ones.

Several studies reported that the decreased efficiency in ethanol production encountered with the high sugar concentration was probably due to osmotic effects (Takeshenge and Ouchi 1995, Roukas 1996, Siqueira *et al.* 2008, Lin *et al.* 2012 and Zohri *et al*. 2012). High sugar concentration and low water activity cause a decrease in yeast cell viability, reduce yeast growth, increase fermentation times, decrease fermentation rates, decrease ethanol production and possibly stuck or sluggish the fermentation process (Graves *et al.* 2007).

Lin *et al.* (2012) observed that when the temperature was increased to 35ºC, the maximum fermentation time was shortened, but a much higher temperature inhibited the growth of the cells and the fermentation significantly declined. This phenomenon may be explained as follows: the higher temperature results in changing the transport activity or the saturation level of soluble compounds and solvents in the cells, which might increase the accumulation of toxins including ethanol inside the cells. Moreover, the indirect effect of high temperature might also be ascribed to the denaturation of ribosomes and enzymes as well as fluidity problems of membranes (McMeckin *et al*., 2002 and Phisalaphong *et al.*, 2006).

Conclusion: The present results showed that *S. cerevisiae* EC1118 was the most potent yeast strain tested for ethanol production using sugar cane molasses and that sugar concentration in these molasses affected the kinetic parameters of the process. The optimum initial sugar concentration for ethanol production and fermentation efficiency was found to be 25% sugar in treated molasses incubated at 35ºC.

Table (1): Fermentation kinetics (F. K.) of bioethanol production from treated and non-treated cane molasses using high ethanol producer yeast strains in batch cultures using cane molasses with 10% initial sugar at pH 4.5 & 35°C after 120 hours**.**

Yeast strains / F. k.	Non-treated cane molasses						Treated cane molasses					
	S.c. EC1118	*S. c.* BY	*S. c.* CY3079	*S. c.* GHM	*K. m.* GU133329	*K. m.* GU133331	*S. c.* EC1118	*S. c.* BY	*S.c.* CY3079	*S. c.* GHM	*K.m.* GU133329	*K. m.* GU133331
TRS (g/l)	3.04	2.71	14.7	3.8	1.15	3.99	5.78	3.61	2.12	3.65	5.01	3.06
E (g/l)	48.61	38.9	40.8	47.7	45.50	46.27	40.82	35.38	33.48	39.05	26.8	38.99
B (g/l)	10.0	6.0	11.0	6.5	7.10	11.00	14.50	13.00	26.50	10.50	21.5	17.00
$Y_{E\ of\ TH}$	95.1	76.1	79.8	93.3	89.04	90.55	79.88	69.24	65.52	76.42	52.4	76.30
V.E.P. (g/l/h)	0.41	0.32	0.34	0.39	0.38	0.39	0.34	0.29	0.28	0.33	0.22	0.32
$Y_{E/CS}$ (g/g)	0.5	0.39	0.47	0.49	0.46	0.48	0.43	0.37	0.34	0.40	0.28	0.40
$Y_{E/IS}$ (g/g)	0.5	0.38	0.46	0.47	0.45	0.46	0.40	0.35	0.33	0.39	0.26	0.38
F_{pH}	4.1	4.1	4.10	4.25	4.10	4.10	4.10	4.10	4.10	4.20	4.00	4.00

Abbreviations: TRS: Total residual sugar (g/l), E: Ethanol concentration (g/l), B: Dry weight of yeast growth (g/l), $Y_{E\ of\ TH}$: Percentage of ethanol yield from theoretical value, V.E.P.: Volumetric ethanol productivity (g/l/h), $Y_{E/CS}$: Ethanol yield over the consumed sugar (g/g), $Y_{E/IS}$: Ethanol yield over the initial sugar (g/g), F_{pH}: Final pH value, *S. c.: Saccharomyces cereviciae, K. m.: Kluyveromyces marixianus***.**

Table (2): Fermentation kinetics (F. K.) of bioethanol production from treated and non-treated cane molasses using high ethanol producer yeast strains in batch cultures using cane

Yeast strains / F. k.	Non-treated cane molasses						Treated cane molasses					
	S. c. EC1118	*S. c.* BY	*S. c* CY3079	*S. c.* GHM	*K. m.* GU133329	*K. m.* GU133331	*S. c.* EC1118	*S. c.* BY	*S. c.* CY3079	*S. c.* GHM	*K. m.* GU133329	*K. m.* GU133331
TRS (g/l)	3.2	5.06	16.4	5.15	4.5	5.5	2.4	3.9	6.8	4.5	3.4	4.4
E (g/l)	56.9	41.8	44.03	41.3	42.2	41.5	70.2	44.6	58.14	52.3	50.2	47.7
B (g/l)	10.8	13.8	12.2	11.05	12.6	11.13	8.7	18.4	12.98	10.6	14.04	13.4
$Y_{E\ of\ TH}$	74.24	54.5	57.4	53.9	55.04	54.2	91.6	58.1	75.9	68.3	65.5	62.23
V.E.P. (g/l/h)	0.47	0.4	0.37	0.34	0.35	0.35	0.6	0.4	0.5	0.44	0.4	0.4
$Y_{E/CS}$ (g/g)	0.41	0.3	0.33	0.3	0.30	0.3	0.5	0.3	0.41	0.4	0.34	0.33
$Y_{E/IS}$ (g/g)	0.38	0.28	0.29	0.28	0.28	0.28	0.5	0.3	0.39	0.4	0.33	0.32
F_{pH}	4.0	4.0	4.2	4.1	4.1	4.3	4.2	4.0	4.2	4.3	4.3	4.4

molasses with 15% initial sugar at pH 4.5 & 35°C after 120 hours.

- Legends as those in Table (1).

Table (3): Fermentation kinetics (F. K.) of bioethanol production from treated and non-treated cane molasses using high ethanol producer yeast strains in batch cultures using cane molasses with 20% initial sugar at pH 4.5 & 35°C after 120 hours.

Yeast strains / F. k.	Non-treated cane molasses						Treated cane molasses					
	S. c. EC1118	*S. c.* BY	*S. c.* CY3079	*S. c.* GHM	*K. m.* GU133329	*K. m.* GU133331	*S. c.* EC1118	*S. c.* BY	*S. c.* CY3079	*S. c.* GHM	*K. m.* GU133329	*K. m.* GU133331
TRS (g/l)	3.34	8.92	17.1	4.1	12.3	6.1	2.2	3.04	7.9	5.8	7.55	5.7
E (g/l)	76.9	57.7	67.79	65.9	75.2	71.6	89.02	65.96	76.3	75.9	78.44	77.9
B (g/l)	10.7	12.3	11.9	12.7	11.7	12.1	10.7	14.98	12.04	13.3	11.64	12.9
$Y_{E\ of\ TH}$	75.2	56.4	66.34	64.5	73.6	70.1	87.1	64.5	74.6	74.3	76.75	76.17
V.E.P. (g/l/h)	0.64	0.5	0.56	0.6	0.6	0.59	0.74	0.6	0.64	0.6	0.65	0.65
$Y_{E/CS}$ (g/g)	0.4	03	0.37	0.3	0.4	0.37	0.5	0.3	0.4	0.4	0.41	0.4
$Y_{E/IS}$ (g/g)	0.38	0.29	0.34	0.3	0.38	0.36	0.5	0.3	0.4	0.4	0.39	0.39
F_{pH}	4.0	4.2	4.20	4.2	4.4	4.1	4.1	4.3	4.2	4.2	4.1	4.4

- Legends as those in Table (1).

Table (4): Fermentation kinetics (F. K.) of bioethanol production from treated and non-treated cane molasses using high ethanol producer yeast strains in batch cultures using cane molasses with 25% initial sugar at pH 4.5 & 35°C after 120 hours.

Yeast strains / F. K.	Non-treated cane molasses						Treated cane molasses					
	S. c. EC1118	*S. c.* BY	*S. c.* CY3079	*S. c.* GHM	*K. m.* GU133329	*K. m.* GU133331	*S. c.* EC1118	*S. c.* BY	*S. c.* CY3079	*S. c.* GHM	*K. m.* GU133329	*K. m.* GU133331
TRS (g/l)	4.12	11.3	11.5	4.9	14.6	7.9	0.83	4.11	8.2	9.8	11.8	6.06
E (g/l)	91.8	71.4	74.6	40.3	55.1	65.95	125.9	77.4	106.7	110.0	89.8	100.76
B (g/l)	10.0	12.0	11.0	11.0	8.00	7.5	13.5	16.5	17.0	20.0	12.00	17.5
$Y_{E\ of\ TH}$	71.8	55.9	58.4	31.5	43.1	51.6	98.5	60.6	83.5	86.1	70.30	78.3
V.E.P. (g/l/h)	0.8	0.6	0.6	0.3	0.46	0.55	1.1	0.7	0.9	0.92	0.75	0.84
$Y_{E/CS}$ (g/g)	0.4	0.3	0.3	0.2	0.23	0.27	0.5	0.3	0.44	0.5	0.38	0.4
$Y_{E/IS}$ (g/g)	0.4	0.29	0.3	0.2	0.22	0.26	0.5	0.31	0.43	0.44	0.36	0.4
F_{pH}	4.1	4.25	4.2	4.2	4.1	4.25	4.3	4.3	4.2	4.3	4.00	4.2

- Legends as those in Table (1).

Table (5): Fermentation kinetics (F. K.) of bioethanol production from treated cane molasses using *Saccharomyces cerevisiae* EC1118 in batch cultures using cane molasses with 30% and 33.3% initial sugar at 35°C & pH 4.5 after 24, 48, 72, 96, 120 &144 hours.

Sugar conc. / F. K.	30% treated cane molasses						33.3% treated cane molasses					
	24	48	72	96	120	144	24	48	72	96	120	144
TRS (g/l)	17.03	11.39	3.8	2.95	2.3	1.4	8.6	5.01	2.5	2.46	2.13	1.6
E (g/l)	32.9	39.94	94.3	80.82	46.8	34.6	6.9	85.06	97.9	92.53	59.7	48.7
B (g/l)	1.1	2.75	4.7	6.15	6.5	6.4	1.0	3.85	4.0	4.55	4.7	4.6
$Y_{E\ of\ TH}$	21.5	26.05	61.5	52.72	30.5	22.6	4.1	49.5	57.5	54.33	35.1	28.6
V.E.P. (g/l/h)	1.37	0.29	2.26	–	–	–	0.3	3.3	0.5	–	–	–
$Y_{E/CS}$ (g/g)	0.12	0.14	0.32	0.27	0.2	0.1	0.02	0.3	0.3	0.28	0.2	0.2
$Y_{E/IS}$ (g/g)	0.11	0.13	0.32	0.27	0.16	0.1	0.02	0.3	0.3	0.28	0.2	0.2
F_{pH}	4.5	4.5	4.5	4.30	4.3	4.3	4.5	4.5	4.5	4.15	4.2	4.2

- Legends as those in Table (1).

Table (6): Fermentation kinetics of bioethanol production from treated cane molasses using *Saccharomyces cerevisiae* EC1118 in batch cultures using cane molasses with 30% and 33.3% initial sugar at 40°C & pH 4.5 after 24, 48, 72, 96, 120 & 144 hours.

Sugar conc. / F. K.	30% treated cane molasses						33.3% treated cane molasses					
	24	48	72	96	120	144	24	48	72	96	120	144
TRS (g/l)	11.9	4.8	3.1	2.5	1.9	1.5	6.9	5.5	5.02	4.6	2.18	1.5
E (g/l)	25.3	81.1	68.2	59.7	54.4	45.4	30.3	79.8	84.6	87.2	84.3	60.6
B (g/l)	2.3	2.8	3.2	3.0	3.4	3.2	1.5	2.0	2.9	4.25	4.65	4.0
$Y_{E\ of\ TH}$	16.5	52.9	44.5	38.9	35.5	29.6	14.1	46.8	49.6	51.2	49.5	28.12
V.E.P. (g/l/h)	1.1	2.3	–	–	–	–	1.3	2.02	0.2	0.11	–	–
$Y_{E/CS}$ (g/g)	0.09	0.3	0.23	0.2	0.2	0.2	0.1	0.24	0.3	0.3	0.25	0.18
$Y_{E/IS}$ (g/g)	0.09	0.3	0.23	0.2	0.2	0.2	0.1	0.24	0.3	0.3	0.25	0.18
F_{pH}	4.5	4.5	4.5	4.2	4.1	4.1	4.5	4.5	4.5	4.15	4.15	4.15

- Legends as those in Table (1).

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