Investigation on Agar.

II. Physico-chemical Properties of Agar and their Influence on the Growth of Microorganisms.

By

Arao Itano and Yasuhiko Tsuji.

[February 10, 1935.]

The previous reports[1),2),3)] dealt with the general review on agar as to the seaweeds from which agar is manufactured and the iodine contents and also the history together with general chemical composition. In this investigation, the physico-chemical properties of agar of different grades I, II and III on the market, were determined and their subsequent influence on the growth of Azotobacter chroococcum, Bacillus subtilis and Saccharomyces cerevisiae was tried. The quality of agar is different by the locality where it is produced, the kind of seaweeds used and the process of manufacturing. Consequently the agar should be carefully chosen according to its use and examined minutely before it is used. Especially for the use in the microbiological investigation, it is necessary to consider the quality of agar as to its physico-chemical properties since a minute quantity of foreign constituent influence their physiological activities.

With the recent increased demand for agar in the scientific and industrial fields, the fundamental investigations have been undertaken, and as the result, the grading of agar is made on some physico-chemical bases instead of the superficial methods which had been employed in the past.

To obtain some definite information as to the influence of agar of different grade upon the microbiological activities, the following investigation was undertaken.

Experimental:

Three different grade of agar, namely I, II and III, manufactured in Hyōgo Prefecture were ground up in powder and subjected to the following tests :

Part I. Determination of Moisture Contents.

1. Moisture contents :

Two grams of agar was dried at 105—110°C. until the weight became constant. The results are given in Table I.

Table I.

Moisture Contents of Agar.

Grade of agar.	Moisture.
	%
I.	20.37
II.	23.24
III.	22.20

Table I indicates that the second grade agar showed the highest moisture contents.

2. Nitrogen contents :

Five to ten grams of agar was taken and analysed for the total nitrogen by KJELDAHL method as usual and obtained the following results :

Table II.

Nitrogen Contents of Agar.

Grade of agar.	Nitrogen in 1 g. air-dried agar.	Nitrogen in 1 g. oven-dried agar.
	(mg.)	(mg.)
I.	0.371	0.466
II.	0.840	1.064
III.	1.862	2.398

As shown above, better the quality of agar is, contained less nitrogen. The second grade agar contained more than twice of nitrogen than the first, and the third grade was more than twice that of the second.

3. Ash contents :

Three grams of agar was burnt in a crucible and the residue was weighed and obtained the following results :

Table III.

Ash Contents of Agar.

Grade of agar.	Ash in 1 g. air-dried agar.	Ash in 1 g. oven-dried agar.
	(g.)	(g.)
I.	0.0282	0.0354
II.	0.0308	0.0401
III.	0.0497	0.0688

Table III indicates, worse the grade contained more ash which suggests that some of the soluble inorganic constituent have been washed down and retained in the third grade.

4. Velocity of Coagulation :

A fixed quantity of agar of different grade as noted in the table, was taken and mixed with 100 cc. distilled water and boiled for an hour with a return condenser and shaking occasionally. While the solution is hot, 10 cc. of it were poured into a test-tube of a definite size, pre-cooled in a constant temperature bath of 45°C. for 20 minutes and transferred to another bath of 30°C. and was examined every hour for the coagulability. The stage from the liquid to coagulation was noted by A, B, C and D, and the degree of coagulation designated by (+) or (−) in each stage. The results are noted in the following tables :

Table IV.

Velocity of Coagulation of 1.5 g. Agar in 100 cc. H_2O.

Time in minutes. / Grade of agar.	1.5	2	2.5	3	3.5
I.	A	B	C	C(+)	D
II.	A	B	C	C(+)	D
III.	A	A	B	C	D(−)

Table IV indicates that no apparent difference was noted between Grade I and II while Grade III was slow.

Table V.

Velocity of Coagulation of 0.7 g. Agar in 100 cc. H_2O.

Time in minutes. / Grade of agar.	3	4	5	6	7
I.	B	C	C(+)	D	D
II.	B	C	C	C(+)	D
III.	A	B	C	C	D(−)

In this concentration, a slight difference was observed between Grade I and II while Grade III was much slower than the others.

Table VI.

Velocity of Coagulation of 0.5 g. Agar in 100 cc. H_2O.

Time in minutes. / Grade of agar.	5	7	9	11	13
I.	A	C	C	C(+)	D
II.	A	C	C	C(+)	D
III.	A	B	C(−)	C(−)	C(+)

Table VI shows that the coagulation rate became much slower in all grades, and no complete coagulation was obtained in Grade III.

Table VII.

Velocity of Coagulation of 0.3 g. Agar in 100 cc. H_2O.

Time in minutes. / Grade of agar.	20	30	40	50	60
I.	B	C	C	C	C
II.	B(−)	C(−)	C	C	C
III.	A	B	C(−)	C(−)	C(−)

In this case, no complete coagulation took place in any grade, and the difference among three grades became more distinct.

Table VIII.

Velocity of Coagulation of 0.1 g. Agar in 100 cc. H_2O.

Time in minutes. / Grade of agar.	30	50	70	90	120
I.	A	A	A	A	A
II.	A	A	A	A	A
III.	A	A	A	A	A

In the above concentration, all grades remained as solution.

5. *Determination of Solubility:*

Placed 0.5 g. agar in 100 cc. H_2O, and immersed in a hot water bath at 90°C. and shaken at different intervals as below, and the solubility was observed.

Table IX.

Solubility of Agar of Different Grade.

Grade of agar. / Hours.	5	10	15	20	25	30	35	40
I.	−	−	−	+	+	+	+	+
	−	−	−	+	+	+	+	+
II.	−	−	−	−	+	+	+	+
	−	−	−	−	−	+	+	+
III.	−	−	−	−	−	+	+	+
	−	−	−	−	−	+	+	+

Notes: (−) indicates not dissolved; (+) completely dissolved.

As Table IX indicates that Grade I was dissolved first which was followed by Grade II and III in the order.

Part II. Determination of Specific Gravity, Electrical Conductivity, Viscosity, Surface Tension and Hydrogen Ion Concentration of 0.1% Agar Solution.

Dissolved 0.1 g. agar in 100 cc. H_2O by shaking one hour and boiling for 10 minutes with a return condenser and left standing at room temperature. Then the following determinations were made and obtained the results noted in Table X:

Table X.

Determination of Physical Properties of Agar.

Properties. \ Grade of agar.	I.	II.	III.
Sp. gr.	1.00012	1.00014	1.00019
Electrical conductivity. (a)	3.536×10^{-5}	3.873×10^{-5}	4.284×10^{-5}
Osmotic pressure. (b)	0.1815	0.4235	0.4719
Viscosity. (c)	1.697	1.576	1.529
Surface tension. (d)	76.0	73.2	70.5
P_H (e)	5.98	6.43	6.32

Notes: a. Determined at 30°C. and expressed in mhos.
b. „ by freezing point method.
c. „ at 45°C. by OSTWALD apparatus.
d. „ by DU NOUY method, in dynes/sq. cm.
e. „ by quinhydrone method.

As the results given in Table X indicate, specific gravity, electrical conductivity and osmotic pressure were larger in the order of I, II and III grade while the viscosity and surface tension were the reverse. Consequently it may be stated that more impurities especially electrolytes were contained in the order of I, II and III grade. Grade I was highest in hydrogen ion concentration while grade II and III were just about the same, and all of them were slightly acidic.

Part III. Determination of Specific Gravity, Electrical Conductivity, Osmotic Pressure, Viscosity, Surface Tension and Hydrogen Ion Concentration of Agar Extracts.

Since the results obtained in Part II indicated that the difference of properties is mainly due to the presence of water soluble matter, it was experimented with the water extract of agar to substantiate the foregoing results.

The extract was prepared by shaking 10 grams of agar in 300 cc. water for 2 hours and filtered through the paper and the determinations were made as in Part II. The results are shown in Table XI.

Table XI.

Determination of Physical Properties of Agar Extracts.

Properties. \ Grade of agar.	I.	II.	III.
Sp. gr.	1.000152	1.000291	1.000434
Electrical conductivity. . (a)	1.401×10^{-5}	2.687×10^{-5}	2.885×10^{-5}
Osmotic pressure. . . . (b)	0.5687	0.6776	0.8470
Viscosity. . . . . (c)	1.140	1.268	1.402
Surface tension. . . . . (d)	70.4	65.2	66.7
P_H . . . . . (e)	6.52	6.86	6.81
P_H . . . . . (f)	6.67	6.88	6.79

Notes: (a), (b), (c) and (d) were determined in the same manner as in case of agar; (e) was determined in the extract which was prepared by shaking 1 g. agar in 20 cc. H_2O for 1 hour, and (f) was tested on agar suspension instead of the extracts.

The results given above, as a whole, indicate the similar tendency as in the previous determination except viscosity which may be due to the colloidal nature. In case of the hydrogen ion concentration, both the extract and emulsion were about the same, Grade I being the highest followed by II and III in the order.

Part IV. Influence of Different Grade of Agar on Bacterial Growth.

By the results obtained in Part I, II and III, it is apparent that there is a considerable difference among the different grade of agar as to the physico-chemical properties.

Since the agar is used in a large quantity in the preparation of solid culture medium, it is naturally expected to influence differently the growth and other activities of bacteria, if different grade of agar is used in their cultivation.

However so for as the authors are aware, no information is available on the subject. Consequently the influence of different grade of agar on Bacillus subtilis, Azotobacter chroococcum and Saccharomyces cerevisiae was undertaken, and the results are reported below.

Experimental.

Grade I and III agar were used in order to bring out the difference plainly if there were any difference in the influence, and the forementioned organisms in our stock were used.

1. Influence on Bacillus subtilis :

The following solid culture media were used :

a.) Albumin agar.

Glucose	1.0 g.
Dipotassium phosphate (K_2HPO_4)	0.5 g.
Magnesium sulfate ($MgSO_4 . 7H_2O$)	0.2 g.
Ferric sulfate [$Fe_2(SO_4)_3 . 9H_2O$]	trace
Egg albumin	0.25 g.
H_2O	1,000.0 cc.
Agar (Grade I or III)	15.0 g.

Difference in Growth of B. subtilis :— By using Grade I and II agar in the above media and made plate culture of B. subtilis and incubated at 30°C. The examination was made everyday for the number of colonies and their size together with the ammonia produced. The ammonia was determined by separating the medium from Petri dish and dissolved after acidifying with sulfuric acid and distilled with PREGEL'S micro-KJELDAHL apparatus and finally by NESSLER'S colorimetric method was used. The results are given in Table XII, XIII and XIV.

Table XII.

Number and Size of Colonies of B. subtilis on Albumin Agar Medium.

Grade of agar. / Days.	I.		III.	
	Number of colonies.	Size of colonies.	Number of colonies.	Size of colonies.
		(1/20 cm.)		(1/20 cm.)
2	51.0	0.34	76.0	0.62
3	77.0	1.26	78.0	1.72
4	74.0	1.74	78.0	2.10
5	—	1.98	—	2.26
6	—	2.10	—	2.56
7	—	2.30	—	2.74

Table XIII.

Number and Size of Colonies of B. subtilis on Nutrient Agar Medium.

Grade of agar. / Days.	I.		III.	
	Number of colonies.	Size of colonies.	Number of colonies.	Size of colonies.
		(1/20 cm.)		(1/20 cm.)
2	77.7	2.45	76.5	3.32
3	76.2	3.10	77.5	4.62
4	73.7	3.52	74.5	5.07

Table XIV.

Ammonia Production of B. subtilis in Albumin and Nutrient Agar Media.

Grade of agar.	Ammonia produced in 100 cc. medium.			
	Albumin agar.		Nutrient agar.	
	(mg. N.)	(mg. N.)	(mg. N.)	(mg. N.)
I.	1.550	1.426	12.505	9.845
III.	1.667	1.587	14.196	11.046

Table XII and XIII indicate that the number of colonies developed was about the same in all media but the size of colonies in the media made of Grade III agar, were larger especially it was very marked on the nutrient agar medium. Also, as Table XIV shows, more ammonia was produced in both media of Grade III agar, and again the difference was very marked in the nutrient agar medium.

2. Influence on Azotobacter chroococcum :

The influence on Azotobacter chroococcum was determined in ASHBY's medium of the following composition :

Mannitol [$C_6H_8(OH)_6$]	10.0 g.
Magnesium sulfate ($MgSO_4 . 7H_2O$)	0.2 g.
Monopotassium phosphate (KH_2PO_4)	0.2 g.
Sodium chloride (NaCl)	0.2 g.
Calcium sulfate ($CaSO . 2H_2O$)	0.1 g.
Calcium carbonate ($CaCO_3$)	5.0 g.
H_2O	1,000.0 cc.
Agar (Grade I or III)	15.0 g.

As in case of B. subtilis, the size of colonies and the nitrogen fixed were determined, and the results are noted in Table XV and XVI.

Table XV.

Number and Size of Colonies of Azotobacter chroococcum in Ashby's Medium.

Grade of agar. / Days.	I.		III.	
	Number of colonies.	Size of colonies.	Number of colonies.	Size of colonies.
		(1/20 cm.)		(1/20 cm.)
2	—	—	36	—
3	179.8	0.99	175.0	1.73
4	183.8	2.02	185.3	2.57
5	191.3	2.53	187.5	3.15
6	—	3.45	—	4.05
7	—	3.67	—	4.62

The results are also given in photograph (Plate XXX).

Table XVI.

Quantity of Nitrogen fixed by Azotobacter chroococcum in Ashby's Medium.

Grade of agar.	Total nitrogen in 100 cc. medium.	Nitrogen fixed in 100 cc. medium if 10 days.
	(mg.)	(mg.)
I.	13.008	11.348
III.	17.455	14.230

The foregoing results indicate that the number of colonies were approximately the same but their size was much larger in Grade III agar medium than in case of Grade I. Also much larger quantity of nitrogen was fixed in Grade III agar.

3. Influence on Saccharomyces cerevisiae :

The following media were used :

A. Laurent's medium :

Ammonium sulfate $[(NH_4)_2SO_4]$	4.71 g.
Magnesium sulfate ($MgSO_4 . 7H_2O$)	0.10 g.
Potassium acid phosphate (KH_2PO_4)	0.75 g.
Maltose ($C_{12}H_{22}O_{11}$)	50.00 g.
H_2O	1,000.00 cc.
Agar (Grade I or III)	15.00 g.

B. Kôdi (malt) extract medium* :

Kôdi (malt) extract	1,000.0 cc.
Agar	15.0 g.

* 300 g. Kôdi extracted in 1,000.0 cc. of water.

The number and size of colonies were determined as in the previous experiments, and the quantity of sugar consumed was determined also in this case. The results are shown in Table XVII, XVIII and XIX.

Table XVII.

Number and Size of Colonies of Saccharomyces cerevisiae on Laurent's Medium.

Grade of agar. / Days.	I.		III.	
	Number of colonies.	Size of colonies.	Number of colonies.	Size of colonies.
		(1/20 cm.)		(1/20 cm.)
2	—	—	29.3	—
3	134.3	0.41	178.8	1.14
4	177.0	0.83	185.5	1.79
5	177.2	1.22	183.5	2.31
6	—	1.38	—	2.43
7	—	1.62	—	2.76

Table XVIII.

Number and Size of Colonies of Saccharomyces cerevisiae on Kodi Agar.

Grade of agar. / Days.	I.		III.	
	Number of colonies.	Size of colonies.	Number of colonies.	Size of colonies.
		(1/20 cm.)		(1/20 cm.)
2	224.0	2.26	225.7	2.66
3	222.5	6.64	223.5	7.45
4	221.0	8.32	225.0	9.70

Table XIX.

Quantity of Sugar consumed in Laurent's and Kodi Media.

Grade of agar.	LAURENT'S.		Kôdi.	
	Residual sugar.*	Sugar* consumed.	Residual sugar*.	Sugar* consumed.
	(g.)	(g.)	(g.)	(g.)
I.	16.414	14.087	13.246	40.919
III.	12.888	18.687	11.411	43.148

* Sugar in 100 cc. medium expressed as maltose.

In all these cases, better results were obtained in Grade III agar medium than Grade I which seems to indicate that there is the same substance which stimulated the physiological activities of organism.

The results obtained thus far may be summarized as follows:

a.) Size of colonies grown on different medium is given by the ratio, dividing the measurement obtained on Grade III agar medium by that of Grade I agar $\left(\frac{\text{Size of Colonies on Grade III agar.}}{\text{Size of Colonies on Grade I agar.}}\right)$

Bacillus subtilis:

Days.	Albumin agar.	Days.	Nutrient agar.
2	3.325	2	1.836
7	1.419	4	2.066

Azotobacter chroococcum:

Days.	ASHBY'S agar.
2	3.050
7	1.584

Saccharomyces cerevisiae :

Days.	Laurent's agar.	Days.	Kôdi extract.
2	3.406	2	1.385
7	2.902	4	1.359

These data indicate plainly that the colonies produced on Grade III agar medium were much larger than those on Grade I agar, especially it was marked at an early stage of growth in albumin, Ashby's and Laurent's media although in the nutrient and Kôdi media, the ratio remained the same.

b.) The similar ratio, $\left(\frac{\text{Grade III agar.}}{\text{Grade I agar.}}\right)$ is given in regard to ammonia production, nitrogen fixation and sugar consumption :

Bacillus subtilis : ammonia production in 10 days.

Albumin agar	1.112
Nutrient agar	1.121

Azotobacter chroococcum : Nitrogen fixed in 10 days.

Ashby's agar	1.253

Saccharomyces cerevisiae :

Laurent's agar	1.326
Kôdi extract agar	1.054

As shown above, in general, the similar tendency as in case of colony was obtained. From these results, it is evident that the quality of agar influences the growth and physiological activities of microorganisms.

Summary :

This investigation was undertaken to ascertain the qualities of different grade of agar and their probable difference in their influence upon the physiological activities of microorganisms, and obtained the following results.

1.) Better the quality of agar contained less total nitrogen and while the velocity of coagulation and solubility is greater.

2.) Both the agar solution and extract, showed smaller specific gravity, electrical conductivity, osmotic pressure and surface tension as the quality was better, but the concentration of hydrogen ions was greatest in Grade I agar.

3.) The better growth of B. subtilis, Azotobacter chroococcum and Saccharomyces cerevisiae was obtained in Grade III agar than in Grade I.

From these results, it is clear that agar differs widely in its properties among the different grade and its use influences the physiological activities of micro-

organisms. Consequently it is necessary to purify the agar obtained on the market before it is used in delicate microbiological investigation.

Literature:

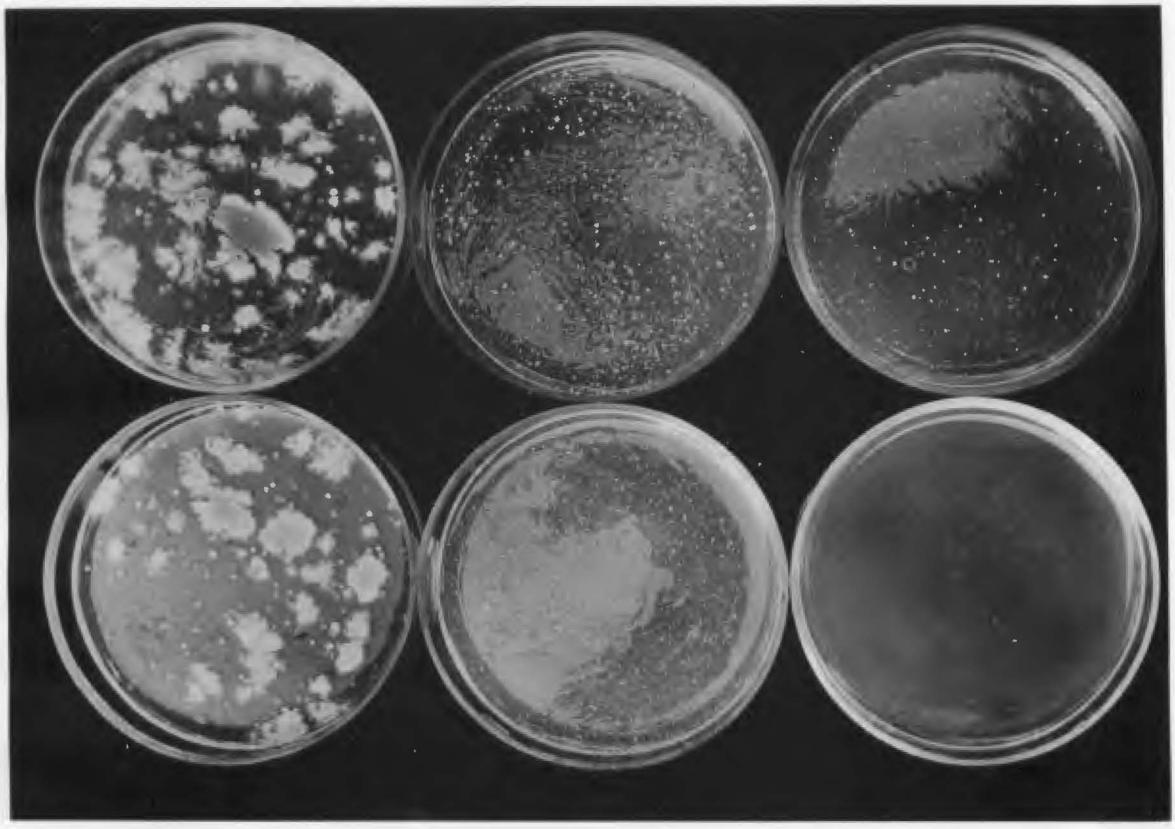
1.) ITANO, A., Reports on General Survey and Investigation on Agar. Berichte d. Ōhara-Inst. f. landw. Forsch., VI: 59—72, 1933.

2.) ———, Investigation on Agar as to its Iodine Content. Proc. Imperial Academy, Japan, IX: 398—401, 1933.

3.) ITANO, A. and TSUJI, Y., Investigation on the Iodine in Agar. (Japanese publication). Nôgaku Kenkyû, XXII: 168—193, 1934.

PLATE XXX.

Colonies on Different Agar Media.



Bacillus subtilis: (4 days old.)

1. Nutrient agar (meat-extract-peptone) Grade III.
4. Ibid. Grade I.

Azotobacter chroococcum: (4 days old.)

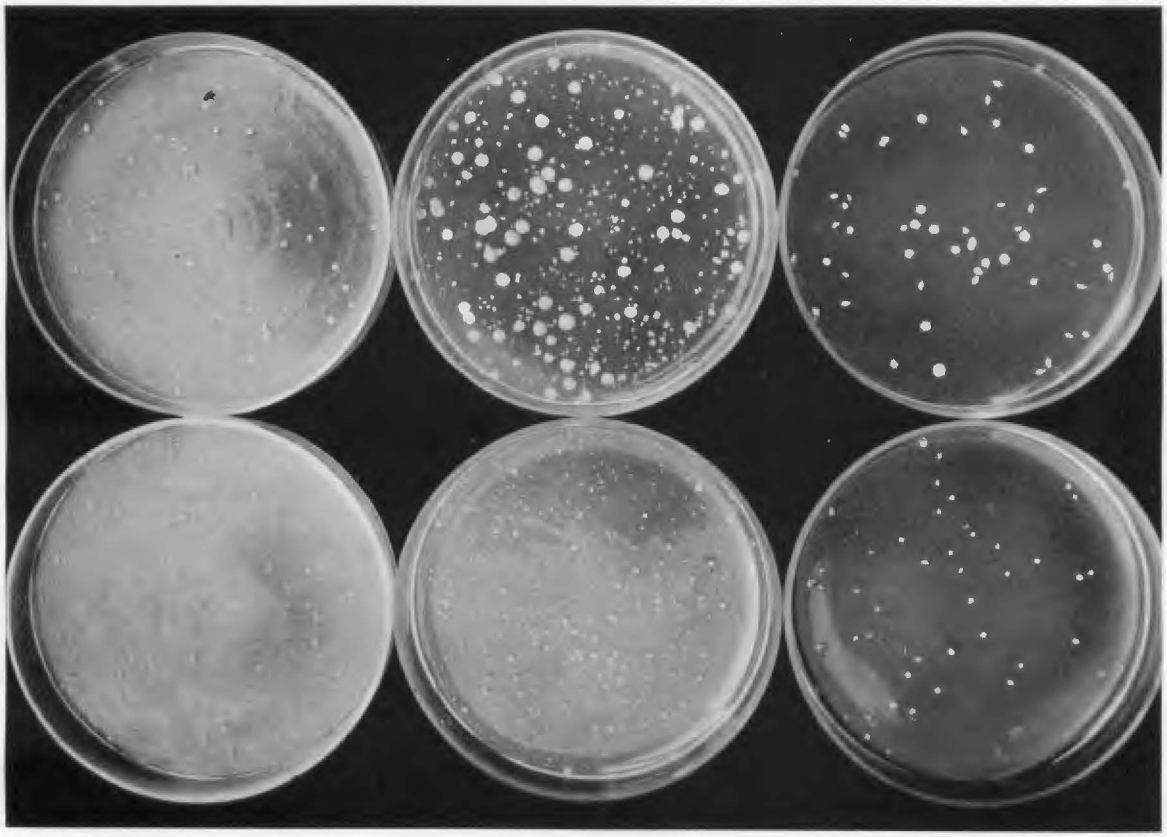
2. Ashby's agar, Grade III.
5. Ibid. Grade I.

Saccharomyces cerevisiae: (4 days old.)

3. Laurent's agar, Grade III.
6. Ibid. Grade I.

PLATE XXXI.

Colonies on Different Agar Media.



Bacillus subtilis: (7 days old.)

1. Albumin agar, Grade III.
4. Ibid. Grade I.

Azotobacter chroococcum: (7 days old.)

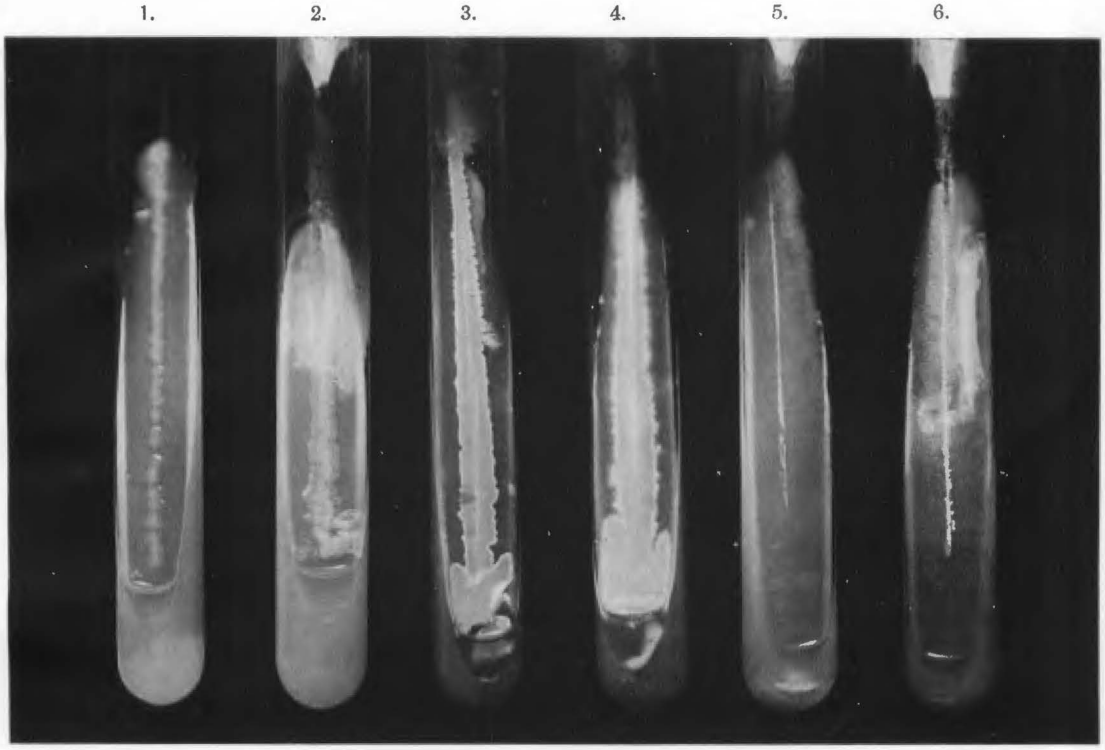
2. Ashby's agar, Grade III.
5. Ibid. Grade I.

Saccharomyces cerevisiae: (7 days old.)

3. Laurent's agar, Grade III.
6. Ibid. Grade I.

PLATE XXXII.

Slant Culture on Different Agar Media.



Azotobacter chroococcum: (5 days old.)

1. ASHBY'S agar, Grade I.
2. Ibid. Grade III.

Bacillus subtilis: (3 days old.)

3. Nutrient agar, Grade I.
4. Ibid. Grade III.

Saccharomyces cerevisiae: (4 days old.)

5. LAURENT'S agar, Grade I.
6. Ibid. Grade III.