THE PRODUCTION OF GLUCONIC ACID AND 2-KETO-GLUCONIC ACID FROM GLUCOSE BY SPECIES OF PSEUDOMONAS AND PHYTOMONAS

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INTRODUCTION

The production of ketogluconic acids from glucose by fermentation methods has been of particular interest to this Bureau as a means of extending the industrial utilization of agricultural products. Our investigations have led to the development of a rapid and efficient process for the production of 5-ketogluconic acid from glucose by *Acetobacter suboxydans*, as heretofore reported (Stubbs *et al.*, 1940). However, we have not found it possible to conduct a fermentation with our strain of *A. suboxydans* on any substrate in such a manner that 2-ketogluconic acid is the major product, as has been reported by Bernhauer and Knobloch (1938). These authors found that a certain strain of *A. suboxydans* gave predominantly 2-ketogluconic acid when cultivated on gluconic acid salts, although when cultivated on a glucose substrate, 5-ketogluconic acid was the principal product. A more recent publication by Bernhauer *et al.* (1940) indicates that such results are dependent on the particular strain of *A. suboxydans* used, for a second strain tested by them yielded 5-ketogluconic acid when cultivated either on glucose or gluconates. Although we have not succeeded in producing 2-ketogluconic acid with *A. suboxydans*, we have found that this product can be very efficiently obtained from glucose by the action of members of a different genus of bacteria. An organism which appeared as a contaminant during an *A. suboxydans* fer-

mentation was isolated, and has since consistently produced 2-ketogluconic acid from glucose or gluconates. A practical process for the production of 2-ketogluconic acid, using this organism, has been described (Stubbs *et al.* 1940). A culture of this organism was sent to Dr. C. B. van Niel, of the Hopkins Marine Station, Pacific Grove, California, who very kindly examined it and classified it as a *Pseudomonas* of the *P. fluorescens* group.

Since ketogluconic acid production by *Pseudomonas* or *Phytomonas* has not been previously reported, an investigation of the fermentation of glucose by bacteria of these genera was undertaken, and the results are presented in this communication.

A literature search revealed the production of gluconic acid by only one species in these genera, *Pseudomonas* (*Phytomonas*) *savastanoi*. The gluconic acid yield of five- to seven-month-old glucose peptone cultures was approximately eighty-five per cent, and no reducing acids could be detected (Alsberg 1911). Conner, Riker and Peterson (1936) reported the occurrence of small quantities of pyruvic and acetic acids in glucose cultures of the hairy root organism (*Phytomonas rhizogenes*).

Of interest in connection with the production of ketones by *Pseudomonas* is the finding of Kluyver, Hof, and Boezaardt (1939) who described the formation of a purple pigment in salted beans. They considered that the pigment was probably derived by autoxidation of triketo-inositol, which was formed by the action of *Pseudomonas beijerinckii* on the meso-inositol present in the beans.

MATERIALS AND METHODS

Our survey studies were conducted in Jena glass gas-washing bottles (Type 101-a), which are constructed with sintered glass false bottoms through which sterile air may be passed, thereby aerating and agitating the cultures. The basic nutrient solution has the composition:

	gms.
Glucose	100
Corn steeping liquor	5
KH_2PO_4	0.6
$MgSO \cdot 7H_2O$	0.25
Distilled water to make one liter.	

Two hundred milliliters of this solution was placed in each Jena glass gas-washing bottle and sterilized at fifteen pounds pressure for one-half hour. After cooling, 2 ml. of a sterile twenty per cent urea solution and 5 grams of calcium carbonate (sterilized dry) were added to each bottle. The cultures were incubated at 30° for eight days, each culture being aerated constantly at the rate of 200 ml. of air per minute.

At the conclusion of each experiment, a determination of calcium in solution was made by precipitation of calcium as the oxalate and subsequent titration with standard $KMnO_4$, in the usual manner.

The original nutrient solutions were analyzed for glucose and the fermented culture solutions for total reducing substances (glucose plus 2-ketogluconic acid) by the copper reduction method of Shaffer and Hartmann (1921). The optical activity of the fermented liquor was also determined after clarification; this value, together with the copper reduction value, permitted the calculation of the concentration of glucose and of 2-ketogluconic acid in the liquors, according to a method described in an earlier publication (Stubbs *et al.* 1940). It should be mentioned here that calcium 2-ketogluconate has a very appreciable negative specific rotation (about $-88°$), in contrast to the considerable dextro-rotation of glucose and the very slight dextro-rotation of calcium gluconate. The exhibition of a levo-rotation by the fermented liquor is, therefore, indicative of 2-ketogluconic acid production. Agreement in the glucose values, as determined by polarimetric observations and copper reduction values, indicates that glucose is the only reducing material present in the cultures represented in table 1.

As a final check, calcium 2-ketogluconate was isolated from the culture liquors of representative species. In order to obtain this salt, the harvested liquors were concentrated at low temperatures to about one-third the original volume, cooled, and the crystalline material filtered off and recrystallized from water. Such purified samples were identified by the comparison of calcium content, copper reduction values and optical rotation with pure known materials. The original identification of 2-ketogluconic acid was made by examination of the methyl ester (Ohle 1937) and

TABLE 1

The production of gluconic acid by species of Pseudomonas and Phytomonas

200 ml. nutrient solution in gas washing bottles contained 20 grams glucose, 5 grams $CaCO_3$, 1 gram corn steeping liquor, 0.4 gram urea, 0.12 gram KH_2PO_4, 0.05 gram $MgSO_4 \cdot 7H_2O$, and one drop oleic acid (antifoam agent). Temperature 30°C., air flow 200 ml. per minute. Duration 8 days.

ORGANISM	GLUCOSE†† CONSUMED	REDUCING VALUE OF FERMENTED LIQUORS‖	OPTICAL ROTATION OF FERMENTED LIQUORS**	TOTAL CALCIUM IN SOLUTION	GLUCONIC ACID FORMED	
					Equivalent to calcium in solution	Yield based on glucose consumed
	grams per 100 ml.			*grams per 100 ml.*	*grams per 100 ml.*	*per cent*
Pseudomonas mucidolens 4685†	9.3	12	+ .68	.81	7.9	78
Pseudomonas mucidolens 4686†	8.4	16	+ .63	.54	5.3	58
Pseudomonas mucidolens 4687†	8.4	29	+1.03	.90	8.8	96
Pseudomonas myxogenes 946†	.9	181	+4.64	0	0	0
Pseudomonas myxogenes 947†	1.4	167	+4.18	0	0	0
*Pseudomonas ovalis**	10.0	7	+1.02	1.02	10.0	92
Bact. flavo-zonatum§ (*Phyto. begoniae*)	1.9	165	+4.54	.11	1.1	52
*Phytomonas campestris**	.2	198		0	0	0
Phytomonas coronafaciens‡	.9	179	+4.33	0	0	0
*Phytomonas michiganense**	.4	192	+4.70	0	0	0
Phytomonas stewarti‡	2.3	157	+4.33	.10	1.0	40
Phytomonas striafaciens‡	9.4	37	+ .70	.84	8.2	80
*Phytomonas syringae**	7.2	51	+1.98	.65	6.4	82
Phytomonas tumefaciens A¶	3.2	144	+4.49	0	0	0
Phytomonas tumefaciens B¶	4.1	124	+4.95	0	0	0
Phytomonas tumefaciens C¶	2.0	150	+4.50	0	0	0

* Received from Dr. N. R. Smith, Bureau of Plant Industry, United States Department of Agriculture.

† Received from the American Type Culture Collection. Culture numbers are theirs.

‡ Received from Dr. Charlotte Elliot, Bureau of Plant Industry, United States Department of Agriculture.

§ Received from Miss Lucia McCulloch, Bureau of Plant Industry, United States Department of Agriculture.

¶ Received from Dr. A. J. Riker, University of Wisconsin.

‖ Expressed in mgm. of copper per ml. of solution.

** Degree of optical rotation of sodium light at 20 to 25° for a 1 dm. tube.

†† Original concentration 9.3 to 10.0 per cent.

the quinoxylin derivative (Ohle 1934). In later cases the methyl ester (m.p. 174–175, Ohle 1937) was prepared to check the identity of the 2-ketogluconic acid.

Gluconic acid was identified in the *Phytomonas* cultures and in the *Pseudomonas ovalis* culture as the phenylhydrazide derivative (Diemair, Bleyer and Schneider 1935).

The rotary aluminum fermenters used in the experiments described here were the vessels constructed in this Bureau several years ago, (Herrick, Hellbach and May 1935), and successfully used in the study of a variety of oxidative fermentations.

EXPERIMENTAL RESULTS

Cultures in gas washing bottles

The results of our study of twelve species (twenty-two strains) of *Pseudomonas* and of eight species (ten strains) of *Phytomonas* are presented in tables 1 and 2. These data show that considerable quantities of gluconic acid or 2-ketogluconic acid are formed in submerged aerated cultures by most of the organisms studied. 2-Ketogluconic acid was the principal metabolic product of *Pseudomonas* species and in many cases represented more than 80 per cent of the glucose consumed. In the culture of *P. fluorescens* (our isolate), it has been previously shown that gluconic acid occurs as an intermediate in the formation of 2-ketogluconic acid (Stubbs *et al.* 1940). That this is also true of the other 2-ketogluconic acid formers is suggested by the fact that the quantity of 2-ketogluconic acid in the harvested liquors is sometimes considerably less than that equivalent to the calcium in solution. Our previous work (Stubbs *et al.* 1940) showed that under the favorable conditions prevailing in the rotary drum fermenters, gluconic acid did not accumulate since it was converted to 2-ketogluconic acid almost as rapidly as glucose was oxidized to gluconic acid. We had therefore concluded that the 2-ketogluconic acid process was not so readily separable into two distinct phases as was the 5-ketogluconic acid fermentation, where no keto-acid was formed until substantially all of the glucose had been oxidized to gluconic acid. The results obtained in our Jena-bottle studies differ from those obtained in the drum fermenters

TABLE 2

The production of 2-ketogluconic acid by species of Pseudomonas

200 ml. nutrient solution in gas-washing bottles contained 20 grams glucose, 5 grams $CaCO_3$, 1 gram corn steeping liquor, 0.4 gram urea, 0.12 gram KH_2PO_4, 0.05 gram $MgSO_4 \cdot 7H_2O$, and one drop oleic acid (antifoam agent). Temperature 30°C., air flow 200 ml. per minute. Duration 8 days.

ORGANISM	GLUCOSE‖ CONSUMED	REDUCING VALUE OF FERMENTED LIQUOR§	OPTICAL ROTATION OF FERMENTED LIQUOR¶	2-KETOGLUCONIC ACID FORMED		CALCIUM		
				Found	Yield based on glucose consumed	Total in solution	Due to 2-ketogluconic acid	
							Grams per 100 ml.	Per cent of total
	grams per 100 ml.			*grams per 100 ml. of solution*	*per cent*	*grams per 100 ml.*		
Pseudomonas aeruginosa†	8.1	114	−4.90	5.8	67	.62	.60	97
Pseudomonas aeruginosa†	7.8	124	−4.90	6.0	71	.64	.62	97
*Pseudomonas fluorescens**	9.3	67	−2.29	3.0	30	.97	.31	32
Pseudomonas fluorescens†	8.7	164	−6.05	7.7	82	.84	.79	94
Pseudomonas fluorescens 948‡	8.3	166	−6.32	7.9	88	.84	.82	97
Pseudomonas fluorescens 949‡	8.3	161	−5.93	7.5	84	.98	.77	79
Pseudomonas fluorescens 142‡	5.8	166	−2.14	4.7	75	.52	.49	93
Pseudomonas fragii 4973‡	9.7	165	−7.77	9.0	86	1.03	.93	90
Pseudomonas graveolens 4683‡	9.7	155	−6.82	8.0	77	.93	.83	89
Pseudomonas graveolens 4684‡	7.5	166	−4.60	6.6	82	.69	.68	98
Pseudomonas mildenbergii 795‡	9.7	186	−9.11	10.5	100	1.08	1.08	100
Pseudomonas ovalis 950‡	7.6	117	−3.09	4.5	55	.63	.46	73
Pseudomonas pavonacea 951‡	5.5	170	−1.77	4.6	77	.58	.48	82
Pseudomonas putida 4359‡	8.7	162	−6.62	8.0	85	.92	.83	90
Pseudomonas schuylkilliensis†	8.6	166	−6.00	7.6	82	.91	.78	86
Pseudomonas vendrelli 7700‡	7.1	168	−4.06	6.2	81	.65	.64	98

* Our isolation.

† Received from Dr. N. R. Smith, Bureau of Plant Industry, United States Department of Agriculture.

‡ Received from the American Type Culture Collection. Culture numbers are theirs.

§ Expressed in mgm. of copper per ml. of solution.

¶ Degrees of rotation at 20 to 25° in a 1 dm. tube.

‖ Original concentration 9.6 to 10.0 per cent.

in that gluconic acid oxidation lags behind glucose oxidation; this circumstance is doubtless due to the facts that the former reaction occurs somewhat more readily, and that the conditions are much more favorable for oxidation in the drum fermenters, where there is a vigorous agitation of the entire contents and a continuous and efficient contacting of the liquid system with the oxygen-containing gas within the fermenter. The operation of the drum fermenters under superatmospheric air pressure also greatly favors other oxidative fermentation reactions, as has been previously shown in a number of cases (Wells *et al.* 1937, Stubbs *et al.* 1940).

It will be noted from table 1 that several strains of *Pseudomonas* and *Phytomonas* produced but little acid. In such cases there were formed considerable quantities of gum, which made the cultures very viscous.

Cultures in rotary fermenters under pressure

In the fermentations by one strain of *Pseudomonas ovalis* and by the active *Phytomonas* species, the oxidation did not proceed beyond the gluconic acid stage, as shown by optical rotation and copper reduction values; consequently the calcium in solution is present as calcium gluconate. Further studies on some of these organisms were conducted in rotary aluminum fermenters to determine whether the more highly oxidizing conditions prevailing therein, as compared with the gas-washing bottles, would result in the production of compounds representing greater degrees of oxidation of the glucose molecule than gluconic acid. The culture medium used was of the same composition as that used in the bottles. The drums, which contained 3300 ml. of nutrient solution, were rotated at thirteen revolutions per minute, and the aeration rate was 1600 ml. per minute. The temperature was held at approximately 25°C., and the pressure was maintained at 30 lbs. per sq. in. (2.11 kgm. per sq. cm.), since these conditions had been found favorable in our earlier studies of *Pseudomonas*. The non-ketogenic strain of *P. ovalis* produced an eighty-two per cent yield of gluconic acid in seventy hours, all the glucose being consumed by this time. Maintaining the culture fifty hours longer under the same conditions did not reduce the gluconic

acid yield appreciably, and did not result in the production of any reducing material.

Phytomonas stewarti, when grown in the rotary fermenter, produced a 47 per cent yield of gluconic acid in 120 hours, with no evidence of ketogluconic acid production. *Phytomonas syringae* gave a 42 per cent yield of gluconic acid in 46 hours, but further oxidized the acid, so that all the material was removed from the solution. The refractive index of the final harvested liquor corresponded to that of water, indicating the complete removal of soluble matter.

Several times during our studies on the production of 2-keto-gluconic acid in rotary fermenters by *P. fluorescens* (our isolate), we have continued operation without change of conditions for as long as two days after the maximum concentration of 2-keto-gluconic acid had been reached. Under such circumstances, the concentration of calcium 2-keto-gluconate slowly and steadily decreases from the maximum value.

Cultures in Erlenmeyer flasks

In order to investigate the metabolism of *Pseudomonas* species when cultivated in non-aerated surface cultures, one strain of *P. ovalis* (the gluconic acid-producing strain) and two strains of *P. fluorescens* were cultured in one-liter Erlenmeyer flasks, each flask containing 200 ml. of nutrient medium of the same composition as that used in the aeration bottles. After 26 days incubation without aeration or agitation these cultures contained only a trace of soluble calcium salts, and had consumed very little glucose, while in eight days the gas-washing bottle cultures had consumed all the glucose, with the formation of soluble calcium salts equivalent to five grams of calcium carbonate. It is thus evident that acid production by these species of *Pseudomonas* is dependent on aeration and agitation of the culture medium, and this circumstance may explain the failure of some previous workers to observe this fermentative activity of these organisms.

DISCUSSION

Within the genus *Pseudomonas*, a wide range of oxidizing ability is known to occur. The work reported here indicates that under

proper cultural conditions, many species of *Pseudomonas* are characterized by a definite but relatively slight oxidizing ability toward glucose. The present study has demonstrated the accumulation of large quantities of gluconic acid and 2-ketogluconic acid in the glucose metabolism of several species of *Pseudomonas* and *Phytomonas*. The genus *Pseudomonas* is shown to contain an interesting group of ketogenic organisms which show similarity to the ketogenic members of the genus *Acetobacter*. They differ from the ketogenic acetic acid bacteria, however, in that after the oxidation of glucose to gluconic acid, the ketogenic *Pseudomonas* strains attack the number two carbon atom of the glucose chain, whereas the fifth carbon atom is the major point of attack in the case of the ketogenic acetic acid bacteria.

In general, it appears that the members of the genus *Pseudomonas* are slightly more active oxidizers than are the species of *Phytomonas* studied, at least under the conditions herein reported. Gluconic acid has been found to accumulate in large quantity in the cultures of several species of *Phytomonas*, but the formation of 2-ketogluconic acid has not been established for this genus.

The finding of considerable quantities of 2-ketogluconic acid in the cultures of *Pseudomonas aeruginosa* (table 2) is of interest in relation to the report of Aubel (1921) that *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*) brought about a fermentation essentially similar to the alcoholic fermentation. In contrast to this, data obtained with our two strains of *P. aeruginosa* show that under our culture conditions the major portion of the glucose is converted to gluconic or 2-ketogluconic acid. This difference might be explained on the basis of special culture conditions such as air supply, agitation, etc., as previously discussed. The findings of Schreder, Brunner and Hampe (1933), and of Neuberg and Kobel (1931, 1932), for *Pseudomonas lindneri*, are essentially similar to those of Aubel, except that Schreder *et al.* found a small quantity of succinic acid in addition to pyruvic and acetic acids. In our examination of *Pseudomonas* and *Phytomonas* culture liquors, we have found no acids other than gluconic and 2-ketogluconic acids. These two acids would be missed in any system of analysis dependent on the extraction of acids by ether, and

2-ketogluconic acid would not be distinguished from residual glucose in the analysis for reducing materials by copper reduction methods, unless polarimetric observations were also made.

It is interesting to note that, under our culture conditions, pigment production was observed only in the cultures of *P. aeruginosa*, which showed a small amount of blue color in the foam above the solution and in the foam traps placed in the outlets.

SUMMARY

The glucose metabolism of *Pseudomonas* and *Phytomonas* species has been studied in submerged aerated cultures. Sixteen strains of *Pseudomonas*, distributed among ten species, produced 2-ketogluconic acid, the yield being in excess of 80 per cent in many cases. One strain of *P. ovalis* produced only gluconic acid.

Four species of *Phytomonas*, of eight species studied, produced gluconic acid in appreciable quantity. None of the *Phytomonas* strains produced 2-ketogluconic acid.

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