Promising Strain of *Acinetobacter* from Soil for Utilization of Gluconic Acid Production

Topraktaki Gelecek Vaadeden *Acinetobacter* Suşunun Glukonik Asit Üretiminde Kullanımı

Research Article

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ABSTRACT

Gluconic acid, a food additive, is used in many foods to control acidity or binds metals such as calcium, iron. *Acinetobacter* sp. WR326, newly isolated from soil possesses high phosphate solubilizing activity and do not require pyrroloquinoline quinone (PQQ) for glucose dehydrogenase (GDH) activity as cofactor In this study the gluconic acid production potential of this bacterium was investigated. Firstly, *Acinetobacter* sp. WR326 was incubated in tricalcium phosphate medium (TCP) with varying glucose concentrations (100, 250, 500 mM), at a temperature of 30°C for 5 days (120 hours). The highest gluconic acid yield (59%) was found at a glucose concentration of 100 mM. Then three different levels of gluconic acid addition to the medium (50, 100, 200 mM) were tested. When *Acinetobacter* sp. WR326 strain was cultivated with a 100 mM glucose and 100 mM gluconic acid the yield increased to 95.27%. In any trials 2 -keto D-gluconic acid, causes problems in processing and purification of the gluconic acid, was not detected in the medium. As a conclusion, *Acinetobacter* sp. WR326 may be considered novel potential bacterial strain for gluconic acid production.

Key Words
Acinetobacter sp. WR32, gluconic acid, fermentation, phosphate solubilizing bacteria, biotechnology.

ÖZ

Glukonik asit asitliği kontrol etmek veya kalsiyum, demir gibi metalleri bağlamak için gıdalarda kullanılan bir katkı maddesidir. Topraktan yeni izole edilen *Acinetobacter* sp. WR326, yüksek fosfat çözme aktivitesine sahiptir ve glukoz dehidrojenaz (GDH) aktivitesi için pyrroloquinoline quinone (PQQ) kofaktörüne gereksinim duymamaktadır. Bu çalışmada bu bakterinin glukonik asit üretme potansiyeli araştırılmıştır. İlk önce, *Acinetobacter* sp. WR326 farklı glukoz derişimlerde (100, 250, 500 mM) 30°C sıcaklıkta 5 gün boyunca (120 saat) trikalsiyum fosfat (TCP) besiyerinde inkübe edilmiştir. En yüksek glukonik asit verimi (%59) 100 mM'lık glukoz derişimi içeren kültürde bulunmuştur. Sonra besiyerine glukonik asit ilavesi üç farklı derişimde (50, 100, 200 mM) araştırılmıştır. *Acinetobacter* sp. WR326 suşu 100 mM glukoz ve 100 mM glukonik asit ilavesinde kültüre alındığında verim %95.27 oranına yükselmiştir. Hiçbir koşulda glukonik asidin işlenmesi ve saflaştırılmasında problemlere neden olan 2-keto D-glukonik asit tespit edilmemiştir. Sonuç olarak, *Acinetobacter* sp. WR326 suşunun glukonik asit üretiminde yeni potansiyel bakteri suşu olarak kullanılabileceği düşünülmektedir.

Anahtar Kelimeler
Acinetobacter sp. WR32, glukonik asit, fermentasyon, fosfat çözücü bakteriler, biyoteknoloji.

Article History: Received: Mar 25, 2017; Revised: May 15, 2017; Accepted: Aug 12, 2017; Available Online: Dec 25, 2017.
DOI: 10.15671/HJBC.2018.203

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INTRODUCTION

Gluconic acid and its salts (sodium and calcium) are widely used in food, pharmaceutical, feed, cement, textile and chemical industries [1,2]. The use of gluconic acid (E 574) as a food additive has been permitted by the European Parliament and Council Directive No. 95/2/EC. Additionally, sodium gluconate is being used in food industry as a substance GRAS (generally recognized as safe) status. The demand for gluconic acid used in different industries is raising day by day [3,4,5]. It is widely accepted that gluconic acid will be one of the top-30 most produced substances from biomass resources in the future [6].

Gluconic acid can be produced by chemical oxidation, electrolysis and fermentation [2,7]. However, microbial fermentation is the method of choice while it is cheaper and no undesired reactions occur [1,7]. Gluconic acid is produced by the fungi genera *Aspergillus, Penicillum, Gliocladium,* and *Endomycopsis,* yeasts such as *Aureobasidium* and by other microorganisms like *Acetobacter* and *Gluconobacter*, and *Pseudomonas* [7]. However, it is mainly produced by strains of *Aspergillus niger* [4,8]. New approaches in studies dealing with gluconic acid production include (i) isolation of new microorganisms, (ii) optimization of the fermentation conditions and (iii) utilization of cheap carbohydrate sources in fermentation [6,9,10].

Dissolved phosphorus is an important factor in plant growth. Phosphate solubilizing bacteria (PSB) convert insoluble phosphate into soluble forms in the soil by releasing organic acids [11,13]. PSB mostly secret gluconic acid produced by direct oxidation of glucose via glucose dehydrogenase (GDH) for the dissolution of hardly soluble phosphorus [12-14]. Although the phosphate solubilizing microorganisms were treated as phosphate solubilizer, they were almost never considered as gluconic acid producer in earlier studies. Ogut et al [12,15] isolated *Acinetobacter* sp. WR326 from soil (Tokat, Turkey) and found out that the *Acinetobacter* sp. WR326 has a high phosphate solubilizing capacity. High phosphate solubilizing capacity is attributed to production of gluconic acid by GDH. For the activation of GDH, pyrroloquinoline quinone (PQQ) as a cofactor is required. Many bacteria such as *Acinetobacter sp. Iwoffii* and some *E.coli* strains synthesize apoquinoprotein (apoGDH), not PQQ [16,17]. As a result, for the oxidation of glucose and phosphate solubilization either the PQQ externally be provided or must be produced in the cell. Ogut et al [12] had shown in their study that *Acinetobacter* sp. WR326 is PQQ-independent phosphate solubilizing microorganism. Considering the high phosphate solubilizing capacity and PQQ independent property of these newly isolated bacteria, in this study, we tested the gluconic acid production potential at varying glucose and gluconic acid concentrations.

MATERIAL and METHODS

Microorganisms and Medium

A 24 h culture of each *Acinetobacter* sp. WR326 (Genbank accession number: HM438960) was used. For the cultivation, TCP medium was used and the composition of the medium was shown in Table 1 [12]. Different levels of glucose (100, 250, 500 mM) and gluconic acid (50, 100, 200 mM) were used in the medium. Glucose, TCP, yeast extract, $MgSO_4.7H_2O$, $CaCl_2$, $Ca_3(PO_4)_2$ were supplied from Merck, Germany. A volume of 50 μL culture was transferred to a 250 mL Erlenmeyer flasks containing 50 mL TCP medium. The microorganisms were cultivated in a shaking incubator (Heidolph D-91126 Schwobach, Germany) set to 30°C temperature and 150 rpm in an aerobic condition. The samples for the HPLC (High performance liquid chromatography) analysis were taken from the 24, 72 and 120 hours old cultures. They were stored at (-) 18°C until analysis.

Experimental Design

At the first stage *Acinetobacter* sp. WR326 was incubated in tricalcium phosphate medium (TCP) with 100, 250 and 500 mM glucose concentrations at a temperature of 30°C for 5 days (120 hours). At the end of incubations the gluconic acid yields were determined and the highest one was recorded to be used at the second stage of experiment.

At the second stage three different levels (50, 100, 200 mM) of gluconic acid were added to medium together with above mentioned glucose concentration and incubated at the same conditions above. Then gluconic acid yields were recorded.

Determination of Gluconic Acid and 2-Keto d-Dluconic Acid

The gluconic acid and 2-keto D-gluconic acid levels were quantitatively analyzed by HPLC (Shimadzu, Japan) using Shodex RS Pak DE-613 (ID 6.0 mm x L 150 mm) column. Elution was performed with an isocratic solvent $HClO_4$ (2 mM) with a flow rate of 0.5 mL min^{-1} at 200 nm wavelength. The column temperature was set at 30°C. The standards of gluconic acid (Merck, Germany) and 2-Keto D- gluconic acid (Sigma Aldrich, U.S.A) were prepared in water. The elution times of the peaks of the samples were compared with the elution time of the standard peak. The limit of detection (LOD) values for 2-keto D-gluconic acid and D-gluconic acid were $3.25x10^{-9}$ mM and $3.85x10^{-9}$ mM, respectively. The samples were centrifuged at 11.000 rpm for 5 minutes (Nüve NF 800, Turkey) after defrosting. The supernatant was diluted to 1:15 and analyzed by HPLC after filtering through 0.45 μm filters.

Statistical Analysis

Triplicate measurements of gluconic acid and 2-keto gluconic acid values were statistically evaluated by analysis of variance (ANOVA) procedure using SPSS statistical software ver. 14.0 (SPSS Inc., Chicago, IL). The differences among the means were compared using Duncan's multiple comparison [18].

RESULTS and DISCUSSION

The medium given in Table 1, was used for cultivation of *Acinetobacter* sp. WR326. Since *Acinetobacter* sp. WR326 is PQQ-independent phosphate solubilizing microorganism we didn't add PQQ. *Acinetobacter* sp. WR326 produced gluconic acid in all experiments (Table 2). In this study, whether *Acinetobacter* sp. WR326 produces 2-keto D-gluconic acid or not was also investigated (Figure 1). Because the keto acids cause problems in processing and purification of the gluconic acid [10]. *Acinetobacter* sp. did not produce 2-keto D-gluconic acid in this study. Hence, it seems to be an advantage that the bacteria used in this study do not produce 2-keto gluconic acid.

Table 1. Composition of tricalcium phosphate medium.

Medium (for 1L)	Stage I: Effect of glucose concentration	Stage II: Effect of gluconic acid concentration
Yeast Extract (g)	0.5	0.5
$MgSO_4.7H_2O$ (g)	0.25	0.25
$CaCl_2$ (g)	0.10	0.10
$Ca_3(PO_4)_2$ (g)	5	5
Glucose (mM)	100, 250, 500	0.1
Gluconic Acid (mM)	-	50, 100, 200

The amounts of gluconic acid in the cultures and the production yields are shown in Table 2. The gluconic acid production yields were calculated by dividing the average gluconic acid amount by the amount of glucose added to the medium and multiplying the result by 100. Different glucose concentrations affected the gluconic acid production potential of the *Acinetobacter* sp. WR326 strain. The gluconic acid levels increased with longer incubation times at all glucose concentration except at 500 mM glucose (Table 2). The glucose concentration of 500 mM was not suitable for the *Acinetobacter* sp. WR326 strain. Similar results were found in a study where the fungus *Aspergillus niger* was cultivated at varying glucose concentrations and varying fermentation conditions [1]. In this study it was found that the gluconic acid decreased when the glucose concentration exceeded 120 g L^{-1}. In our study the highest yield of gluconic acid was determined in the medium containing 100 mM glucose. So we used a glucose concentration of 100 mM thereinafter. When the 120 hours incubation period is taken into consideration, the highest gluconic acid level (21.10 g mL^{-1}) were determined in the medium containing 250 mM glucose (Table 2). However, the production efficiency at that concentration was low.

As the addition of the gluconic acid to the medium probably solubilized the tricalcium phosphate to release phosphorus ions, which are readily usable in bacterial energy metabolism [12-14]. The effects of gluconic acid addition into the medium were also investigated.

In the calculation of yield, the effect of gluconic acid addition was considered. Different gluconic acid addition levels to the medium (50 mM,

Table 2. The amount and yield of gluconic acid.

	Glucose Concentration (mM)	Incubation Period (hour)	Gluconic Acid Amount ($g.L^{-1}$)	
			Mean±SD*	Yield%
Stage 1	100	24	7.07 ± 0.018^{f}	39.26
		72	10.40 ± 0.007^{d}	57.70
		120	10.63 ± 0.009^{c}	59.00
	250	24	7.80 ± 0.009^{e}	17.32
		72	12.49 ± 0.006^{b}	27.73
		120	21.10 ± 0.036^{a}	46.85
	500	24	6.12 ± 0.008^{g}	6.80
		72	6.10 ± 0.011^{g}	6.77
		120	5.92 ± 0.003^{h}	6.57

	Glucose Concentration (mM)	Gluconic Acid Concentration (mM)	Incubation Period (hour) Mean±SD*	Gluconic Acid Amount ($g.L^{-1}$)	
				Yield (%)	
Stage 2	100	50 72 120	24	5.49 ± 0.047^{i}	30.45
			9.26 ± 0.030^{g}	51.42	
			11.79 ± 0.028^{d}	65.44	
	100	100 72 120	24	8.17 ± 0.024^{h}	45.33
			13.70 ± 0.068^{c}	76.05	
			17.16 ± 0.031^{a}	95.27	
	100	200	24	9.33 ± 0.155^{f}	51.57
			72	10.65 ± 0.018^{e}	59.10
			120	16.67 ± 0.055b	92.53

* Means followed by the same letter within each column are not significantly different and values are means ± standard deviations of measurements of three measurements ($p<0.05$).

100 mM and 200 mM) influenced the production positively. The yield of culture media without gluconic acid was 59%. However, following 100 mM gluconic acid addition to culture media the yield increased to 95.27% (Table 2). The determined yield value is the same with the theoretical value determined using *Aspergillus niger* [7].

CONCLUSION

Acinetobacter sp. WR326 bacteria resulted in the highest yield of gluconic acid production at 100 mM of glucose and 100 mM of gluconic acid concentrations. This newly isolated and PQQ independent bacterium may be considered a novel potential gluconic acid production source according to our findings. The PQQ independency of *Acinetobacter* sp. WR326 may be assumed as a benefit in terms of economy during the large scale production of gluconic acid. Currently, fungi genera seem the potential producer of gluconic acid in the microbial fermentation route. This strain of *Acinetobacter* isolated from soil may be an alternative source for microbial fermentation route. 2 -keto D-gluconic acid was not detected in the medium. This may another advantage as the

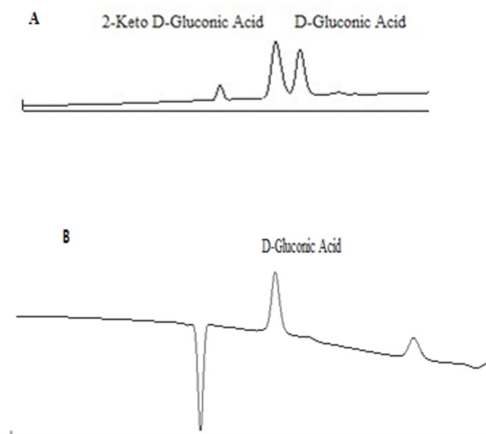

Figure 1. A: Chromatogram of D-gluconic acid; 2-Keto D-gluconic acid standards. B:Chromatogram of culture by Acinebacter sp. WR326 at glucose concentration of 100 mM. Column Shodex RSpak DE-613 (6.0 mm I.D. x 150 mm). UV-200 nm. Mobil Phase: 2 mM HClO4 a.q., flow rate: 0.5 mL/min. Column oven: 30ºC.

keto acids may cause problems in the processing and purification steps of the gluconic acid. Based on increasing demand for gluconic acid, investigation of alternative cultivation conditions are necessary to optimize the feasible gluconic acid production process.

ACKNOWLEDGMENT

This work was supported by Selçuk University No: 13401094.

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