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ORIGINAL PAPER

High cell density production of *Deinococcus radiodurans* under optimized conditions

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Abstract *Deinococcus radiodurans* is a bacterium being investigated for mechanisms of extreme radiation resistance and for bioremediation of environmental radioactive waste sites. In both fundamental and applied research settings, methods for large-scale production of *D. radiodurans* are needed. In this study, a systematic investigation was carried out to optimize *D. radiodurans* production at the 20-L fermentor scale. In defined medium, the phosphate buffer typically used was found to be inhibitory to *D. radiodurans* growth, and caused cell aggregation. Substitution of HEPES and MOPS buffers for phosphate buffer improved *D. radiodurans* growth characteristics. Several antifoaming agents were investigated to support large-scale production with submerged aeration, and the defoamer KFO 673 was chosen based on its ability to prevent foaming without affecting *D. radiodurans* growth. The conventional undefined rich medium tryptone/glucose/yeast extract (TGY) maximally supported *D. radiodurans* growth to an OD_{600} of 10. Using a 'design of experiments' approach, we found glucose, Mg and Mn to be critical in supporting high-density growth of *D. radiodurans*. The optimal pH and temperature for *D. radiodurans* growth in large-scale preparations were 7.0 and 37°C, respectively. Growth was carried out in a 20-L fermentor using the newly developed media under the optimal conditions. With addition of 10 g/L glucose, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 5 μM $MnCl_2$ into TGY media, an OD_{600} of 40 was achieved.

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Keywords *Deinococcus radiodurans* · Growth media · Growth conditions · Design of experiment · Fermentor

Introduction

Deinococcus (*Micrococcus*) *radiodurans* was first reported in 1956 by Arthur W. Anderson, isolated from cans of ground beef which had been exposed to ionizing radiation ($\sim 2 \times 10^4$ Gy) [1]. *D. radiodurans* is a non-pathogenic, gram-positive, red-pigmented, spherical bacterium which typically grows as a diplococcus or tetracoccus. *D. radiodurans* is one of the most radiation resistant organisms yet discovered, capable of surviving acute doses of γ-radiation that exceed 15 kGy [10], and growing under chronically-delivered γ-radiation (60 Gy/h) [8]. The molecular mechanisms underlying extreme radiation resistance in *D. radiodurans* have been the subject of basic research for decades [7, 15], but remain poorly defined. The prevailing hypotheses of extreme radiation resistance in *D. radiodurans* fall into at least two categories [7, 15, 16]: (1) a subset of uncharacterized genes encode functions that greatly enhance the efficiency of DNA repair; and (2) non-enzymic Mn(II) complexes present in *D. radiodurans* protect enzymes from oxidation during irradiation, with the result that repair systems survive and function with far greater efficiency than in sensitive bacteria.

In applied research, *D. radiodurans* is a prospective candidate for bioremediation of radioactive environmental waste sites, based on its ability to grow and functionally express cloned genes during exposure to chronic γ-irradiation [4, 5]. For example, *D. radiodurans* has been engineered to completely degrade aromatic hydrocarbons (e.g., toluene) and reduce toxic metals (e.g., Cr^{6+}, Hg^{2+}) in the presence of ^{137}Cs [5]. In the United States, buried Cold

War radioactive waste (3×10^6 m^3) has contaminated about 7×10^7 m^3 of surface and subsurface soils and about 3×10^{12} dm^3 of groundwater [8]. Numerous reports have defined the growth requirements of *D. radiodurans* in small batch cultures of undefined rich medium or defined minimal medium [6, 20]. Studies on large-scale production of *D. radiodurans*, however, are lacking and needed to meet the anticipated demand for this organism in field applications [2, 4, 5, 11, 14], and for studies now dedicated to identifying and characterizing the radioprotective cytoplasmic constituents of this organism [9].

Since the discovery of *D. radiodurans*, TGY (1% bacto tryptone, 0.1% glucose, and 0.5% yeast extract) medium [1] has been the staple growth substrate used to culture this bacterium. Cultures in a phosphate-buffered medium have been used to define the minimal growth requirements of *D. radiodurans*, applying an OFAT approach (changing only one factor at a time) [18, 19], where Embden–Meyerhof–Parnas pathway substrates (e.g., fructose), nicotinamide, $MnCl_2$, and a rich source of amino acids yielded a doubling-time at 32°C of approximately 18 h, and a maximum cell density of OD_{600} 0.9. Currently, the highest growth-yield reported for *D. radiodurans* in defined medium is OD_{600} 5.0, which used MOPS-buffered media containing mixtures of vitamins, amino acids, and salts, and a doubling-time of 2.6 h [12]. However, large-scale production of *D. radiodurans* by fermentation has not been reported. In this study, an efficient method was developed for large-scale preparations of *D.* radiodurans using a 20-L fermentor. A systematic Design of Experiment (DOE) approach yielded conditions which supported growth of *D. radiodurans* to an OD_{600} of 32 in 21 h, producing 250 g of fully viable cells from 14-L broth. Cell extract derived from the harvested cell pellets was used in experiments aimed at characterizing the composition of small molecules implicated in protecting proteins from oxidative modifications caused during irradiation.

Materials and methods

Chemicals

Antifoams DF 204 and DF 10P were obtained from BASF Performance Chemicals (USA). AF 289, antifoam C, PPG 2000, Na_2HPO_4, NaH_2PO_4, K_2HPO_4, KH_2PO_4, MOPS, $MnCl_2$, and Na-glutamate were purchased from Sigma-Aldrich (Milwaukee, WI). KFO 673 was purchased from Emerald Performance Material, LLC (Cheyenne, WY). HEPES free acid was purchased from MP Biomedicals, Inc (Solon, Ohio). Tris buffer was purchased from KD Medical (Columbia, USA). Yeast extract (YE) and tryptone was purchased from BD (Sparks, USA). Glucose and $MgSO_4 \cdot 7H_2O$ were purchased from Mallinckrodt Baker (Phillipsburg, USA).

Strains

Deinococcus radiodurans (ATCC BAA-816) was kindly provided by Dr. Michael Daly at the Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, Maryland.

Shake flask studies

TGY medium containing 10 g/L tryptone, 5 g/L YE, 1 g/L glucose, was used as basic medium. For buffer selection, either pH 7.0 potassium phosphate, HEPES, MOPS, or Tris buffer was added to the basic media at 25 mM, with unbuffered TGY medium used as a control. For antifoam selection, different antifoams were added to the basic media, with TGY media as control. Each shake flask contained 50 ml of medium, and the shaker incubator was set at 32°C, 220 rpm.

For DOE, buffered TY medium containing 10 g/L tryptone, 5 g/L YE, 50 mM HEPES, was used as basic medium. 50 mM HEPES served as extra buffer capacity to support high cell density growth. Four components (10 g/L glucose, 10 g/L Na-glutamate, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 5 μM $MnCl_2$) were added to corresponding shake flasks with basic media based on the DOE design (Table 1). Each shake flask contained 20 ml of medium, and the shaker incubator was set at 32°C, 220 rpm. JMP5 (SAS) was used as the software for the full factorial design and data analysis.

pH and temperature study

Optimized medium (10 g/L tryptone, 5 g/L YE, 10 g/L glucose, 10 g/L Na-glutamate, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 5 μM $MnCl_2$, 50 mM HEPES) was used for temperature and pH optimization. Each shake flask contained 25 ml of medium, and the incubator was set at 220 rpm. Initial pH values studied of 7.0, 7.25, 7.5, and temperatures of 32°C and 37°C were studied. Initial growth rate was measured based on the data collected from the first 6 h of growth.

20-L fermentation

A 20-L BioFlo 4500 fermentor (New Brunswick Scientific, Edison, NJ) with a working volume of 14-L was used for large-scale fermentation. The fermentor was batched with 10 g/L tryptone, 5 g/L YE, 5 g/L glucose, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 5 μM $MnCl_2$, 25 mM HEPES. Additional glucose was added to 5 g/L at 16 h post inoculation. Temperature was controlled at 37°C, pH was controlled at 7.0 with 25% H_2SO_4, and dissolved oxygen (DO) was

Table 1 Shake flask results for DOE

SF No.	Glucose	Glutamate	Mn	Mg	OD_{600}
1	–1	–1	–1	–1	9.95
2	–1	–1	–1	1	11.2
3	–1	–1	1	–1	12.6
4	–1	–1	1	1	20.1
5	–1	1	–1	–1	15.2
6	–1	1	–1	1	21.6
7	–1	1	1	–1	14.0
8	–1	1	1	1	20.7
9	1	–1	–1	–1	10.7
10	1	–1	–1	1	15.6
11	1	–1	1	–1	16.7
12	1	–1	1	1	42.2
13	1	1	–1	–1	19.0
14	1	1	–1	1	38.6
15	1	1	1	–1	19.5
16	1	1	1	1	39.3

–1 Means no addition, 1 means addition: 10 g/L for glucose, 10 g/L for Na-glutamate, 0.5 g/L for $MgSO_4 \cdot 7H_2O$, 5 μM for $MnCl_2$

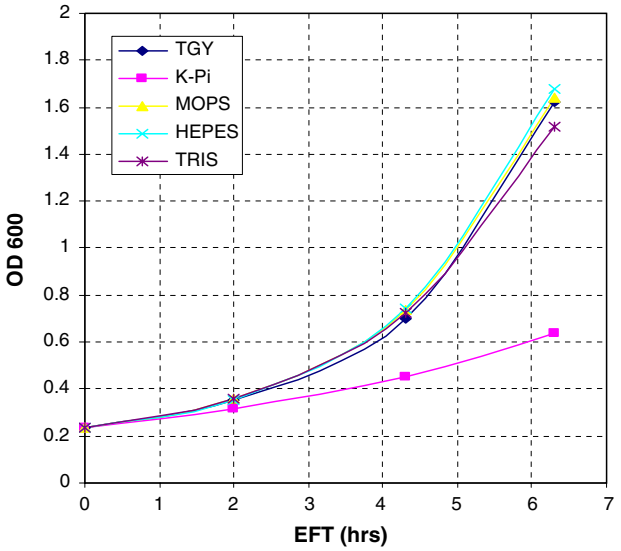

Fig. 1 *Deinococcus radiodurans* growth profile with different buffers in shake flasks. Conditions: either pH 7.0 potassium phosphate, HEPES, MOPS, Tris buffer was added to the basic media at 25 mM, with unbuffered TGY medium as control. Each 250 ml shake flask contained 50 ml of medium, and the shaker incubator was set at 32°C, 220 rpm

controlled at 30%. BioCommand Plus software from NBS was used for data collection. Samples were taken at timed intervals. Cell density was measured at 600 nm (OD_{600}) and supernatants were analyzed for glucose, ammonia, acetate and lactate using BioProfile 300B (NOVA Biomedical, Waltham, USA). Cell paste was harvested using a CARR Pilot continuous centrifuge with a rotation speed of 15,325 rpm (20,000 *g*) with a pump feed-rate of 250 ml/mim.

Results and discussion

Buffer selection

In order to support high cell density growth, media should be properly buffered for pH control. Four buffers were tested and the results are shown in Fig. 1. *D. radiodurans* growth profiles were similar when it grew in HEPES, MOPS, and Tris buffered TGY, or unbuffered TGY. However, potassium phosphate buffer was inhibitory to *D. radiodurans* growth. *D. radiodurans* doubling time was increased in potassium phosphate buffered TGY from 2.0 to 4.2 h, and final OD was about half of *D. radiodurans* grown in unbuffered TGY medium (11.8 compared to 6.7). Some cells grown in potassium phosphate buffered TGY medium aggregated rather than having their normal diplococcus or tetracoccus morphology. Aggregation in *D. radiodurans* and other bacteria is a sign of oxidative stress, and is manifested in *D. radiodurans* when Mn is limited; soluble Mn(II) is readily bound and precipitated by phosphate. Since the OD measurement is affected by cell size, a supplemental experiment was carried out to measure cell dry weights in addition to OD readings. For cells grown in unbuffered TGY media, 139 mg cell dry weight per one OD_{600} reading was generated from each 24 h 1 L culture. Similarly, for cells grown in potassium phosphate buffered TGY media, the ratio was 155 mg cell dry weight per one OD_{600} reading. Cell aggregation only affected the OD reading slightly; therefore, the OD profile shown in Fig. 1 still represented the real cell growth. The inhibitory effect of potassium phosphate buffer on *D. radiodurans* growth was confirmed. In this work, HEPES was chosen as the buffer for further study. As a note, the pH did not change significantly during the first 6 h of incubation in the shake flask. The final pH of the unbuffered TGY medium after 24 h culture was approximately 8.0; and the pH was 7.6 for HEPES buffered medium. The pH did not have over effects on *D. radiodurans*' morphology in liquid culture; between pH 7.0–8.0 in the shake flask, cells were grouped predominantly as diplococci, with a few tetracocci also observed.

Antifoam selection

Because large-scale fermentations are carried out in fermentors with submerged aeration, an effective antifoam is needed to prevent foaming. Six kinds of antifoaming agents from different manufacturers were tested in shake flasks. As shown in Fig. 2, DF 204 and AF 289 were inhibitory to *D. radiodurans* growth. PPG 2000 and DF 10P did not affect *D. radiodurans* growth. Based on initial growth rate,

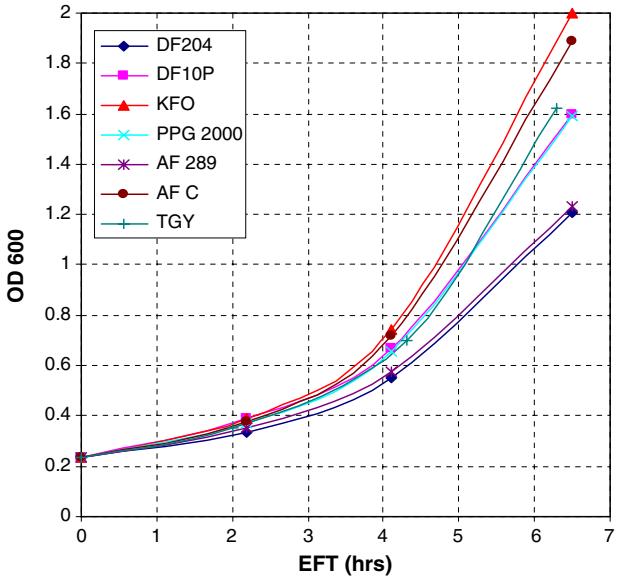

Fig. 2 *Deinococcus radiodurans* growth profile in shake flasks with different antifoam agents. Conditions: different antifoam agents were added to the basic media with TGY media as control. DF 204: 200 µl/L. DF 10P: 100 µl/L. KFO 673: 100 µl/L. PPG 2000: 50 µl/L. AF 289: 100 µl/L. 25% AF C (antifoam C): 200 µl/L. Each 250 ml shake flask contained 50 ml of medium, and the shaker incubator was set at 32°C, 220 rpm

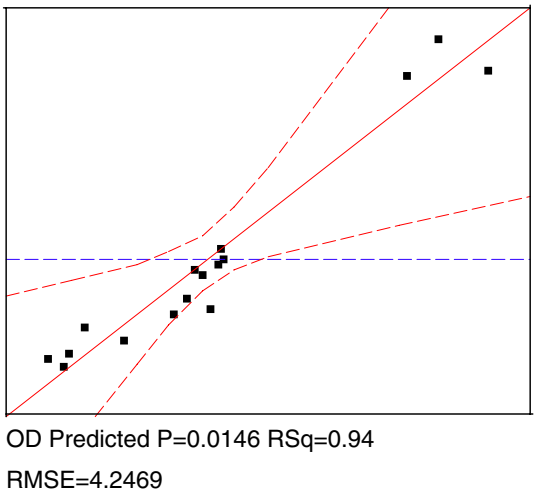

Fig. 3 Design of experiment model accuracy shown by high R^2 value and low P value

KFO 673 and antifoam C improved *D. radiodurans* growth, when compared to the TGY control culture. KFO 673 defoamer (100 µl/L) was chosen as the antifoam for large-scale fermentation.

Media improvement using DOE (full factorial)

The use of statistical experimental design is frequently used to replace the conventional 'one-variable-at-a-time' approach for media optimization in fermentation and cell culture process development [3, 13, 17], because it generates results rapidly and can account for interactions between media components. In order to have a balanced medium with enough nutrients to support high cell density growth, a full factorial design was applied in this study comprising four components at two levels. The four components were glucose, glutamate, Mg, and Mn. Glucose and glutamate were chosen, because they had been shown to improve final OD (data not shown). Mn was chosen, because *D. radiodurans* is reported to be highly dependent on this transition metal [12, 20]. Mg was chosen, because it is needed for DNA, RNA synthesis in all known cell types. Table 1 shows the results from the DOE experiment, which included 16 shake flasks. TY media (10 g/L tryptone, 5 g/L YE, 50 mM HEPES) without any addition had a final OD of 9.95. The highest OD of 42.2 was achieved by addition to buffered TY medium with glucose, Mg and Mn. Data analysis was performed by JMP5 software and the accuracy of the DOE model is shown in Fig. 3. The P value of 0.015 shows the significance of the model, and R^2 of 0.94 (94%) shows that the majority of variation is accounted for by the model. Table 2 was generated by JMP5, and shows P values (Prob > F) for each component and their interactions. The lower the P value, the higher the significance of the addition on cell density. The P values for glucose, glutamate, Mn, and Mg were 0.007, 0.035, 0.052 and 0.003, respectively. Addition of glucose and Mg had a higher impact on cell density than addition of glutamate and Mn. Among the interactions between components, the interaction between glucose and Mg was the most significant with a P value of 0.037. The higher impact of glucose and Mg addition is also shown in the desirability profile generated by JMP5 (Fig. 4). The desirability plots with or without glutamate or Mn is flat, indicating a low impact of glutamate or Mn on final OD. The DOE model was able to predict the final OD profile with different additions, as shown in the cube plot in Fig. 5. The highest ODs (35–42) were obtained by the addition of both glucose and Mg plus either Mn or glutamate. Because aggregation of cells was observed in the microscope when Na-glutamate was added to the media, the fermentation medium was prepared without Na-glutamate. The relationship between aggregated and healthy cells is not clearly established, so it was determined that maintaining *D. radiodurans*'s diplococcus or tetracoccus morphology would be preferred. The optimized medium contained glucose, Mg, and Mn, but not Na-glutamate.

Effect of pH and temperature

A variety of different pH and temperature conditions have been used for *D. radiodurans* growth in other studies. In one example, Holland and colleague [12] found that

Table 2 Factors which had high impact on cell density

Source	*F* ratio	Prob > *F*
Glucose	20.15	0.007
Glutamate	8.27	0.035
Mn	6.48	0.052
Mg	29.11	0.003
Glucose and glutamate	0.64	0.461
Glucose and Mn	2.05	0.211
Glutamate and Mn	7.03	0.045
Glucose and Mg	7.97	0.037
Glutamate and Mg	0.62	0.468
Mn and Mg	2.59	0.168

D. radiodurans grew better at pH 7.0 than pH 6.4. In the same study, a temperature of 35°C was used for growth, because it resulted in lower heat shock protein expression than growth at 37°C, although *D. radiodurans* grew fastest at 37°C. In another example, *D. radiodurans* grew at pHs ranging between 7.5 and 8.0 when the growth temperature was 32°C [20]. In this study, the initial growth rates were studied at 32°C and 37°C with initial pH set at 7.0, 7.25, or 7.5. The result is shown in Table 3. At 32°C, *D. radiodurans* had the highest growth rate at pH 7.0 with a doubling time of 2.3 hr. At pH 7.0, *D. radiodurans* grew faster at 37°C than at 32°C with a doubling time of 1.9 h. Therefore, pH 7.0 and temperature 37°C were chosen as the conditions for 20-L scale fermentation.

20-L scale fermentation

Growth of *D. radiodurans* in a fermentor has not been reported. Because large amounts of cells were needed to purify the putative radioprotective agents from the cells, a 20-L fermentor was used to produce *D. radiodurans* at pH 7.0 and 37°C with the optimized medium, as shown in the section "Materials and methods".

Figure 6 shows the DO and agitation profile during the fermentation and with cell density measured at 600 nm. A maximum of 390 rpm was needed in the fermentor to keep the DO reading at 30% (dissolved O_2 probe was calibrated to 100% before inoculation with medium saturated with air). *D. radiodurans* cells, which were red-pigmented,

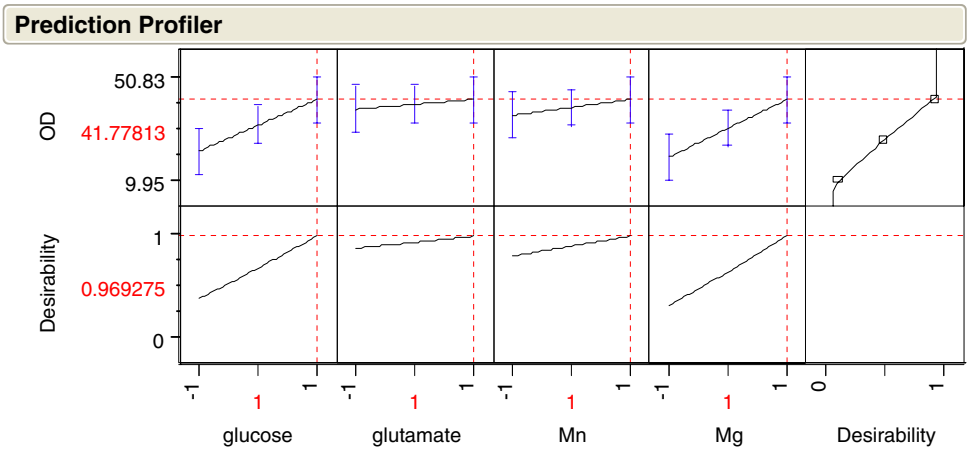

Fig. 4 Desirability predicted by DOE model

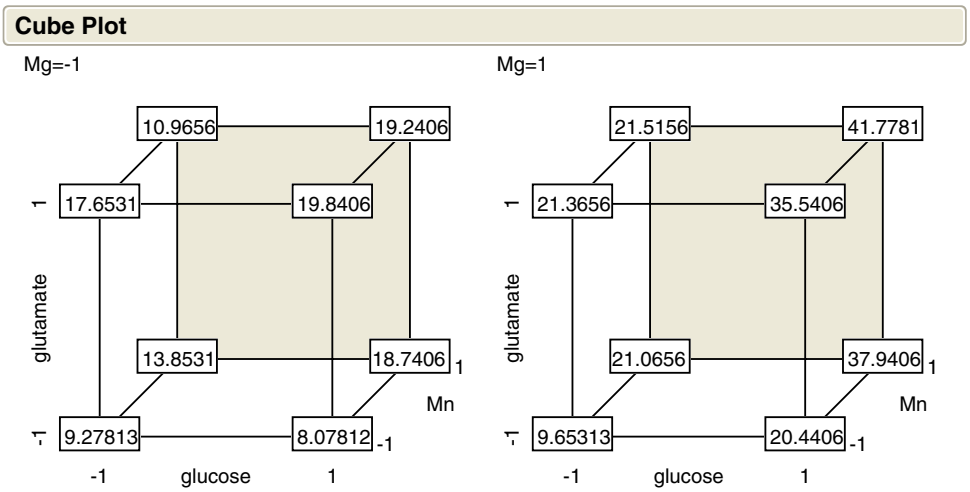

Fig. 5 Condition predicted by DOE to support high cell density growth

Table 3 Effect of pH and temperature on *D. radiodurans* growth rate

pH	Temperature (°C)	μ	t_d (h)
7	32	0.3046	2.3
7.25	32	0.2915	2.4
7.5	32	0.2717	2.6
7	37	0.37	1.9

Conditions: Optimized medium (10 g/L tryptone, 5 g/L YE, 10 g/L glucose, 10 g/L Na-glutamate, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 5 μM $MnCl_2$, 50 mM HEPES) was used. Initial growth rate was measured based on the data collected from the first 6 h of growth

were harvested at late exponential phase with an OD_{600} of 32, and 250 g of cell paste was collected from 14-L broth, approximately 18 g/L. Protein-free cell extract derived from the harvested cell pellets was tested to confirm its radioprotective properties, and was utilized in a study to identify the composition of small molecules responsible for protecting proteins during irradiation. Therefore, the optimized medium did not affect *D. radiodurans'* resistance to radiation or the level of its red pigmentation.

Figure 7 shows the offline data measured by BioProfile 300B. Little acetate and lactate were produced during the fermentation. Glucose was not utilized until late exponential phase (OD = 26). *D. radiodurans* preferred other carbon source in YE and tryptone over glucose. *D. radiodurans* started to use glucose when those preferred carbon sources were depleted. Ammonia was continually produced while cell density was increasing, suggesting that *D. radiodurans* was using amino acids as a primary carbon energy source. When glucose consumption started, ammonia production decreased. 25% H_2SO_4 was added as required to maintain pH at 7.0, as shown in the pH profile of Fig. 6. Both YE and tryptone contain large amounts of glutamate, 160 mg per g of YE and 185 mg per g of tryptone. Since 10 g/L of tryptone and 5 g/L of YE were added in the fermentation, the total glutamate concentration reached 2.65 g/L in the media. Glutamate can be oxidized by glutamate oxidase to form 2-oxoglutarate, which then enters the TCA cycle and provides the energy for cell maintenance and growth (KEGG Pathway). During glutamate oxidation, one ammonia molecule is released. Holland and colleague [12] simplified their defined media with only glutamate and serine as amino acids, consistent with the importance of glutamate in *D. radiodurans* metabolism. During the 20-L fermentation, 26 OD were generated during amino acid consumption, and 6 OD were generated during glucose consumption. As expected, more growth was obtained during amino acid consumption than during glucose consumption [20].

Due to time constraints, it was not possible to fully define a composition for an optimized medium which was completely consumed by the end of fermentation. Based on the consumption of glucose and glutamate in 20-L fermentation, the ratio of glucose and glutamate was 3:2.7 (g/L). The optimal ratio of all components could be determined through another design of experiment: response surface design. The question of whether or not supplementation of the medium specifically with glutamate was not examined. As reported by Venkateswaran et al. [20], *D. radiodurans* is dependent on exogenous amino acids, and the extent of growth is dependent on the total amino acid concentration, not the composition of the amino acid pool. Therefore, other amino acids likely could be substituted for glutamate during high-density growth of *D. radiodurans*. It should be noted, Na-glutamate was not used in the fermentor because it caused cell aggregation; cell-clumping caused by 10 g/L Na-glutamate (59 mM) likely was the result of the high concentration of Na, not glutamate. Thus, other forms of

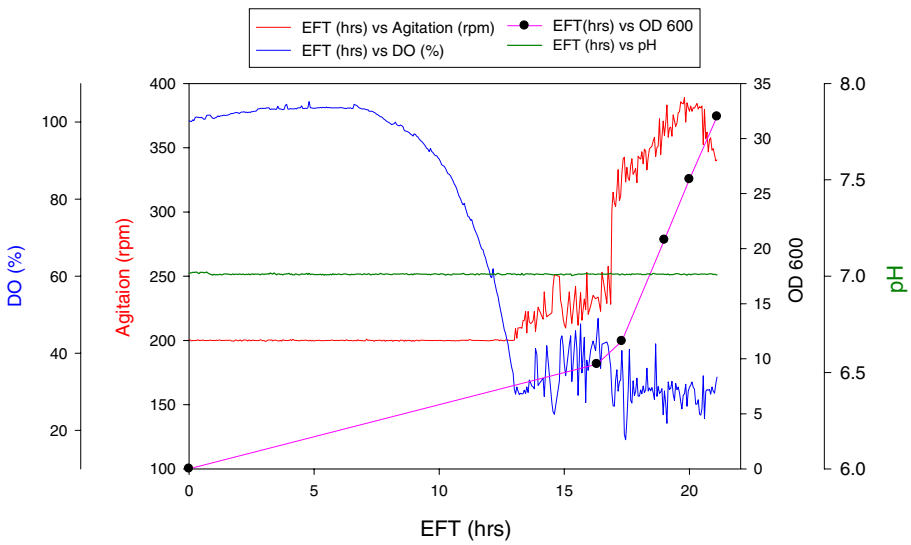

Fig. 6 Online data and OD_{600} profile from 20 L fermentor. The fermentor was batched with 10 g/L tryptone, 5 g/L YE, 5 g/L glucose, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 5 μM $MnCl_2$, 25 mM HEPES. Additional glucose was added to 5 g/L at 16 h post inoculation. Temperature was controlled at 37°C, pH was controlled at 7.0 with 25% H_2SO_4, and dissolved oxygen (*DO*) was controlled at 30% (dissolved O_2 probe was calibrated to 100% before inoculation with medium saturated with air)

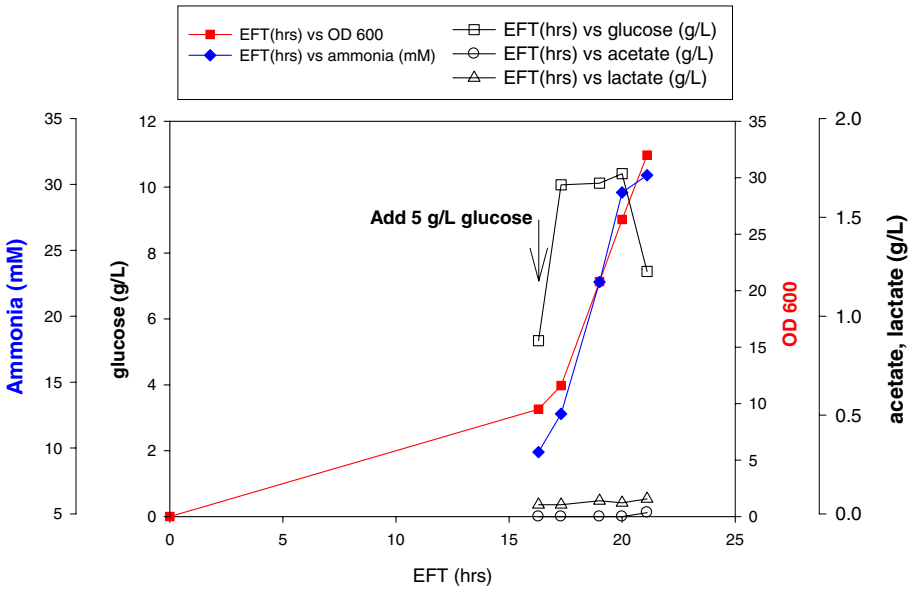

Fig. 7 Offline data and OD profile from 20 L fermentor. Supernatants from samples were analyzed for concentration of glucose, ammonia, acetate and lactate using BioProfile 300B

glutamate, such as ammonia glutamate or glutamic acid could be used, but this was not tested.

Conclusion

Deinococcus radiodurans was successfully produced at the 20-L fermentor scale with optimized medium and optimal fermentor operating conditions. A maximum cell density of 40 OD_{600} units was achieved under these conditions, and 250 g of cell paste was collected from one 14-L fermentation broth. Potassium phosphate buffered medium has been widely used in characterizing the growth requirements of *D. radiodurans* [18, 19]. In this study, HEPES- and MOPS-buffered media preparations supported superior growth yields compared to media containing potassium phosphate, which slowed the growth of *D. radiodurans* and promoted cell clumping. A systematic DOE approach is a powerful strategy to optimize growth media for maximal cell growth in a short period of time. Using this approach, and within just one set of experiments, *D. radiodurans* cell density production was increased from 10 OD_{600} to 40 OD_{600}. Addition of Mg and glucose was found to be critical for achieving the highest cell densities. Off-line medium analysis for 20-L fermentation found that glucose was not utilized until the late stage of fermentation. Based on the fact that ammonia was continuously produced during the fermentation, it was concluded that amino acids were the preferred carbon source under rich media conditions; this finding is consistent with previous studies [20]. One outcome of the systematic experiments presented here are insights into formulating a practical defined medium for large-scale production of *D. radiodurans*, which could facilitate applications of this organism in bioremediation field-settings.

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