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Nutrient consumption and production of isoflavones in bioreactor cultures of *Pueraria lobata* (Willd)

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Abstract: *This paper reports the successful culture of Pueraria lobata (Willd.) suspension cells in a bioreactor. In vitro culture of this Chinese herb has potential as an alternative production method for industrial applications. Calli of P. lobata obtained from leaf explants were cultured in a 5.0 L bioreactor for two weeks. During this period, the pH of the medium declined from 5.8 to 4.5. By the end of the run about 70% of the sugars and reducing sugars and about 50% of nitrate was consumed. Almost 70% of inorganic phosphate and about 80% of the iron was exhausted. The bioreactor results indicated an isoflavone yield of 328.9 μg/ml, with an increase of about 1.77 fold. The yield of puerarin increased about 2.42 fold and reached 73.4 μg/ml in the bioreactor culture.*

Key words: *Pueraria lobata (Willd.), Bioreactor culture, Nutrient consumption, Puerarin, Isoflavones*

Introduction

Pueraria lobata (Willd.) Ohwi (common name kudzu) is a perennial legume plant, wildly distributed in the countries of eastern Asia. Its root is a traditional Chinese herbal medicine, named Gegen or Kakkon in Chinese and Japanese medicine respectively (Zhu, 1998). Its root has been used for the treatment of angina pectoris, hypertension, deafness, optic nerve atrophy or retinitis and is widely used as an antipyretic, antidiarrhetic, diaphoretic and antiemetic substance as well (Liu *et al*., 1999). The isoflavones are the main active compounds of *P. lobata* and isolation of these from the roots and determination of chemical structure by HPLC were reported previously (Cao *et al*., 1999; Yu *et al*., 2002). These include puerarin, daidzein, daidzin and daidzein-42, 7-diglucoside (Cao *et al*., 1999). Recent investigations demonstrate that puerarin, the main active compound in *P. lobata,* is an effective antioxidant and is also effective against glutamate excitotoxicity on cultured mouse cerebral cortical neurons (Guerra *et al*., 2000).

Clonal propagation of *P. lobata* has been studied for some time as a potential industrial alternative to produce these isoflavonoids (Liu *et al*., 1999; Yu and Li, 1999). HPLC analysis for isoflavonoid content indicated that micropropagated plants produced the main isoflavonoid compounds, similar to leaves and roots of intact *P. lobata* plants (Barbara, 2003). Calli of *P. lobata* were induced from different plant organs (Liu *et al*., 1999). While in the process of *in vitro* culture, the content of isoflavones in different organ calli was determined (Barbara, 2003; Chen *et al*., 2004; Matkowski *et al*., 2004) and it appeared that root calli synthesised most puerarin, followed by leaf calli. Furthermore, in suspension culture *P. lobata* also produced isoflavones (Liu and Li, 2002; Zhang and Li, 2003a) and manipulation of the organic supplementation and elicitor used in these cultures could increase isoflavone production. Additionally, 0.2% casein hydrolysate (CH) significantly promoted the growth of cell cultures and the accumulation and release of puerarin and total isoflavones (Li and Zhang, 2006; Zhang and Li, 2003b). The use of dimethyl sulphoxide (DMSO), Tween-20 and Triton X-100 also positively influenced the yield of secondary metabolites, by increasing the permeability of cell and vacuole membranes and hence inducing release of secondary metabolites. Tween-20 or Triton X-100 increased cell biomass as well as the release of puerarin and isoflavones. However, Triton X-100 treatment promoted mainly the accumulation of total isoflavones in cell cultures, affecting a 45.6% increase (Zhang *et al*., 2004).

The advantage of using bioreactors in culture processes is that culture parameters, such as pH, temperature, dissolved oxygen concentration and agitation can be monitored and controlled. Thus it is possible to study cell behavior by determining growth kinetics, nutrient uptake and metabolite production. Such information is essential to identify the key factors for optimization studies (Gorret *et al*., 2004). The scaling up of production of puerarin and other isoflavones in bioreactor has not been done earlier, and we initiated investigations on suspension cultures of *P. lobata* in bioreactors.

Materials and Methods

Plant materials: Leaves of *P. lobata* growing in the Botanical Garden in South China Normal University were sequentially rinsed in water, 70% EtOH for 30 seconds and 0.1% $HgCl_2$ for 7 min, and finally rinsed three times in sterile water. Excised leaves were inoculated on Murashige and Skoog medium (Murashige and Skoog, 1962), supplemented with 1mg l^{-1} naphthalene acetic acid (NAA)

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2 mg l^{-1} benzylaminopurine (BA) and 30 g l^{-1} sucrose, to induce callus formation. After 5 days, white yellowish colored callus clumps were formed at the edge of the leaf explants. Pieces of callus were excised and transferred to the same medium for subculture. The callus was subcultured every 15 days. After a sequence of 6 subcultures, a light-yellowish colored, friable callus was obtained, which was the optimal material for initiation of suspension cultures. Callus (1.5 g fresh wt.) was transferred to a 150 ml flask. After three times suspension culture, the callus (100g fresh wt.) was transferred into a bioreactor containing the liquid medium.

Fermentation was carried out in LI bioreactors (LI, BIOF-2005 China). The full volume was 5 litre, with 3.5 litre working volume. Temperature, aeration and agitation were regulated at 25°C, 99% DO and 120 rpm, respectively and the continuous airflow rate was set at 100 l h^{-1}. The bioreactor was equipped with a 2.5 cm diameter entrance as feed inlet. Culture medium was removed with a hosepipe.

The kinetics of puerarin and other isoflavone production and nutrient exhaustion in bioreactor cultures of *P. lobata* (Willd.) were studied as follows. For fresh weight (FW) determination, excess water was gently removed from callus and the callus was weighed. The calli and the liquid medium were subsequently dried in an oven at 60°C for 24 hr and the dry weight (DW) of calli was determined. To determine nutrient consumption, the liquid medium was filtered using a sieve with a mesh size of 45 μm (made in Shanghai, China). The pH, stirring velocity and oxygen supply was monitored with digital meters in real time.

Sugar content: The total sugar concentration was detected by the anthrone reagent method, taking glucose solution as a standard (Zhang, 1998). A 1ml culture solution (3% sugar content) of *P. lobata* from the bioreactor was placed into one 500 ml volumetric flask, and distilled water added to 500 ml. The diluted solution was transferred into a clear tube, anthrone reagent added (5 ml) and mixed. After the samples were boiled for 10 min and were chilled, the absorbance was determined at 625 nm using a spectrometer and the A_{625} sucrose concentration curve was calculated.

The residual sugar was determined with the 3, 5 dinitrosalicylic acid method (Zhang, 1998). 1ml culture solution of *P. lobata* from the bioreactor was placed into a volumetric flask and distilled water added to 25 ml. One ml of diluted solution was taken out and 3,5 dinitrosalicylic acid 1.5ml was added and mixed. The flasks were boiled for 5 min, chilled to room temperature in a beaker filled with cold water, distilled water added to 25 ml and mixed. At 540 nm the absorbance was detected, and the content of residual sugar in the sample was established from the standard curve.

Nitrogen content: This was detected by the salicylic acid method (Zhang, 1998). One ml of culture solution of *P. lobata* was removed from the bioreactor and ion free water was added to 25 ml. 0.1 ml of the diluted solution was mixed with 0.4 ml 5% salicylic acid - sulphuric acid. 9.5 ml 8% NaOH was added to the solution. The absorbance was detected at 410 nm, and the content of nitrate nitrogen in the sample was established from the standard curve.

Ammonium: It was detected by the Nessler's reagent method (Zhang, 1998). One ml culture solution of *P. lobata* from the bioreactor was added to ion free water to 25 ml. 0.1 ml of the diluted solution was added to 1ml potassium - sodium tartrate and mixed. After standing for 5 min, 1% gum acacia (1 ml) was added, the mixture shaken and Nessler's reagent (1.5 ml) was added, the solution was diluted to 25 ml and mixed. The absorbance were detected at 420 nm and the content of ammonium nitrogen in the sample was established from the standard curve.

Phosphorus content: It was determined by molybdenum blue method (Zhang, 1998). One ml culture solution of *P. lobata* from the bioreactor was diluted to 5 ml. One ml of this was dropped into a 25 ml volumetric flask, to which was added 1ml sulphuric acid-ammonium molybdate and 1ml reducer, the mixture shaken up and boiled for 30 min. After chilling, water was added to 25ml, mixed and the absorbance detected at 650 nm. The phosphorus content in the sample was established from the standard curve.

Iron: The determination of iron ion was done as described by Li *et al.* (2003). One ml culture solution of *P. lobata* from the bioreactor was diluted to 15 ml. Ten ml of the diluted solution was placed in a tap funnel, with 1ml hydrochloric acid and 2 drops 0.1N potassium permanganate added and mixed. 5ml isoamyl alcohol and 5 ml 20% potassium thiocyanate were then added in the tap funnels, shaken for 30 sec. and the layer of water removed. The layer of isoamyl alcohol was transferred to a tube, and the absorbance was detected at 485 nm. The iron content in the sample was then established from the standard curve.

Measurement of total isoflavones, puerarin and daidzein: Total isoflavones, puerarin and daidzein were extracted and determined from bioreactor cultures following oven drying (60°C) and crushing of the dried crude culture. Dry powder (25 mg) was blended with 30 ml 95% EtOH and placed in a water bath at 70°C for 6 hr. After cooling, the crude extract was diluted with 95% EtOH to 50 ml. Samples (1.0 ml) of this extract were diluted with distilled H_2O to 25 ml. As control, 1.0 ml 95% EtOH was diluted with distilled water to 25 ml. Absorbance of samples were measured at 250 nm using a UV-VIS spectrophotometer (UV 2100, Shimadzu, Japan). The linear regression equation found was $y = 0.0287x - 0.0081$; $R^2 = 0.9939$, where x is the concentration of total isoflavonoid (mg l^{-1}), y is E_{250}, which was determined by measuring a series of concentrations of standard puerarin samples. For HPLC analysis, 100 mg dry powder each sample was extracted in 1.0 ml absolute EtOH for 24 hr. Extracts were combined, filtered and diluted with absolute EtOH to 5 ml.

The HPLC (Gold System, Beckman) was used as follows: column, Ultrasphere ODS (4.6 mm×250 mm); eluant, MeOH: H_2O (50: 50); flow rate, 1.0 ml/min; detection, E248 nm. The concentration of puerarin was calculated according to the linear regression equation $y = 70029x + 24172$; $R^2 = 0.9988$. The equations were calculated using the puerarin standard. The concentration of daidzein was calculated according to the linear regression equation $y = 122215x + 7725.1$; $R^2 = 0.9934$, where y is the peak area, x is the concentration of daidzein. Standard puerarin and daidzein were purchased from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). The results presented are the means of three independent experiments.

Results and Discussion

***P. lobata* growth curve in bioreactor culture**: *P. lobata* showed the typical "s" type growth curve in bioreactor culture (Fig. 1). When the initial density of 3.6g l^{-1} dry mass was incubated, the lag phase of cell growth (d 1 to d 2) was 2 days. During the exponential growth phase, from day 3 to day 12, cells reproduced rapidly. After 10 days, the growth rate of *P. lobata* slowed down, but the dry mass increased. At day 12 the dry weight reached a peak, when cell growth entered the stationary phase and the dry weight accumulation stabilized. The bioreactor culture time of *P. lobata* was longer, compared with shaker culture: the lag phase was increased by 2 days and the exponential phase was also extended. However Zhang and Li (2003a) found that the dry weight of cell increased at a similar rate in the two culture systems. Also, compared with callus culture of *P. lobata* on solid medium, the bioreactor culture time was found to be shorter (Chen *et al.*, 2004).

Nutrient exhaustion in the bioreactor: Because the airflow rate was maintained at a high rate of 100 litre h^{-1}, the DO concentration remained between 97% and 99%. During the first 4 days, the medium pH declined slightly from 5.80 to 4.50, then steadied between 4.4 and 4.6 (Fig.2). However in alfalfa cell culture, in a bioreactor, McDonald and Jackman (1989) found that the pH first declined and then rose. This was also reported by Hohe and Reski (2002). These changes in medium pH were probably related to the absorption and utilization of the ammonium sources from the medium (McDonald and Jackman, 1989; Liu, *et al.*, 1999; Gorret *et al.*, 2004).

When the calli of *P. lobata* were induced and cultured on the media with varying pH, the induced rates of calli were significantly affected (Fig. 3). At pH 6.5, the rate of callus induction was 28.92% and as the pH declined the induction rate increased, peaking at pH 5.0 (99.12%). After 20 days at pH 6.5 the calli were small, brown and solid at the edge of the leaf segments

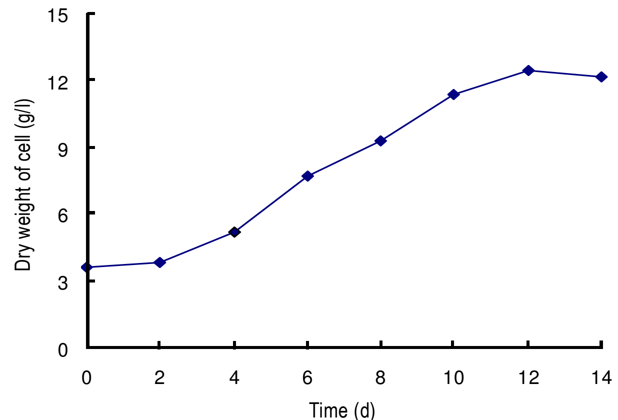

Fig. 1: Curve of dry weight accumulation in bioreactor culture of *P. lobata* leaves

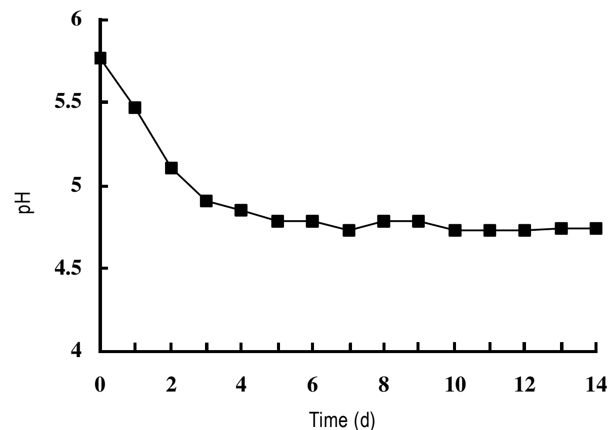

Fig. 2: pH changes in the liquid medium of *P. lobata*

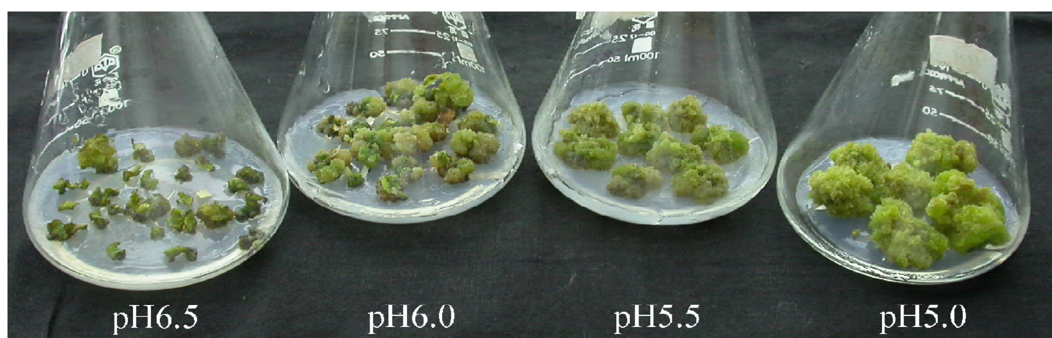

Fig. 3: Callus induction and growth of *P. lobata* at different pH levels

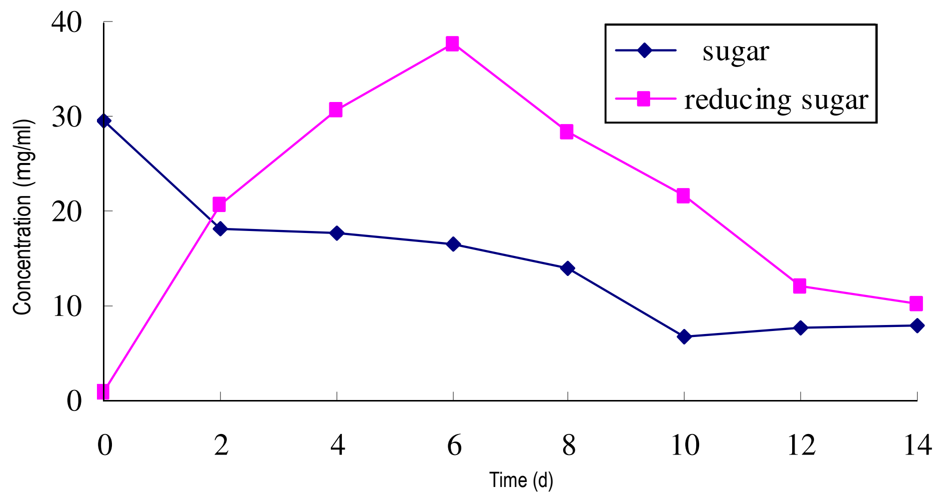

Fig. 4: Residual and reducing sugar concentration changes in bioreactor culture medium of *P. lobata*

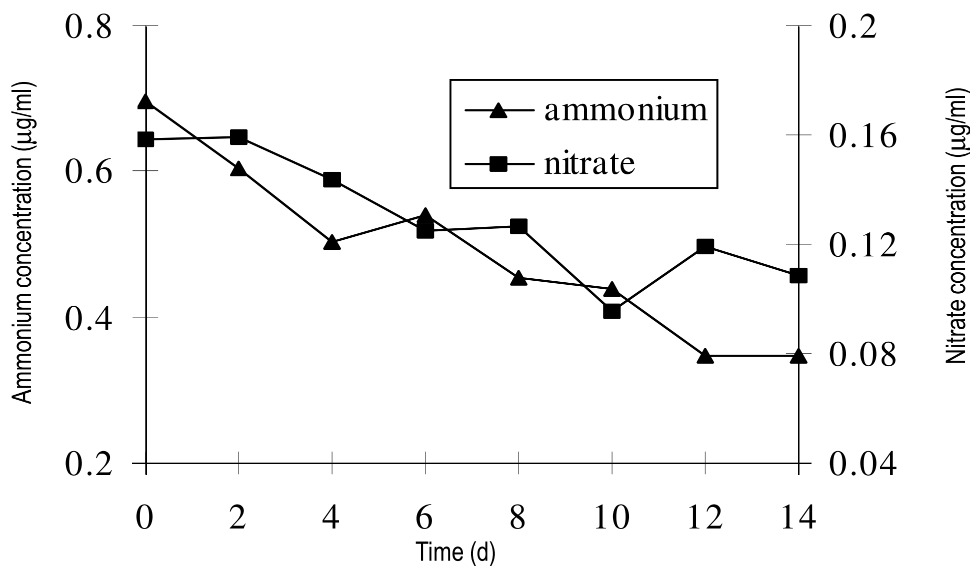

Fig. 5: Changes in ammonium and nitrate concentration in bioreactor culture medium of *P. lobata*

(Fig. 3). At pH 5.0, the calli of whole leaf segments were large, green and friable. This suggests that lower pH was better for cell growth.

The results of residual sugar and reducing sugar exhaustion in bioreactor culture are shown in Fig.4. After 14 days of culture, about 80% of the sugar was utilized. As total sugar was gradually depleted during the growth cycle, the residual sugar concentration increased. The reason was that sucrose in the medium was first hydrolyzed. The concentrations of glucose and fructose increased as the culture moved from the initial to the exponential growth phase. This suggests that the extracellular hydrolysis of most of the sucrose occurred during this period (Liu *et al.*, 1999; Gorret *et al.*, 2004). The accumulation of dry weight was related to the accumulation of carbohydrate in the medium. The onset of dry biomass reduction coincided with the complete removal of sugar from the medium. A lower uptake rate was observed at the late phase. At the initial growth phase, there was almost no reducing sugar in the medium. During cell culture, the sugar concentration fell, but the reducing sugar concentration increased. At the exponential growth phase (linear phase), the reducing sugar concentration declined. This pattern was also found in alfalfa, southernwood and oil palm in suspension culture or in bioreactor culture (McDonald and Jackman, 1989; Liu, *et al.*, 1999; Gorret *et al.*, 2004). Indeed, the carbon balance showed that only 20% of carbon used was converted into biomass indicating that 80% was converted into non-measured products such as CO_2, ethylene, or intracellular storage substances (Gorret *et al.*, 2004).

Nitrogen was another important source for cell growth, and the mol concentration ratio was 1:2 in MS medium. The

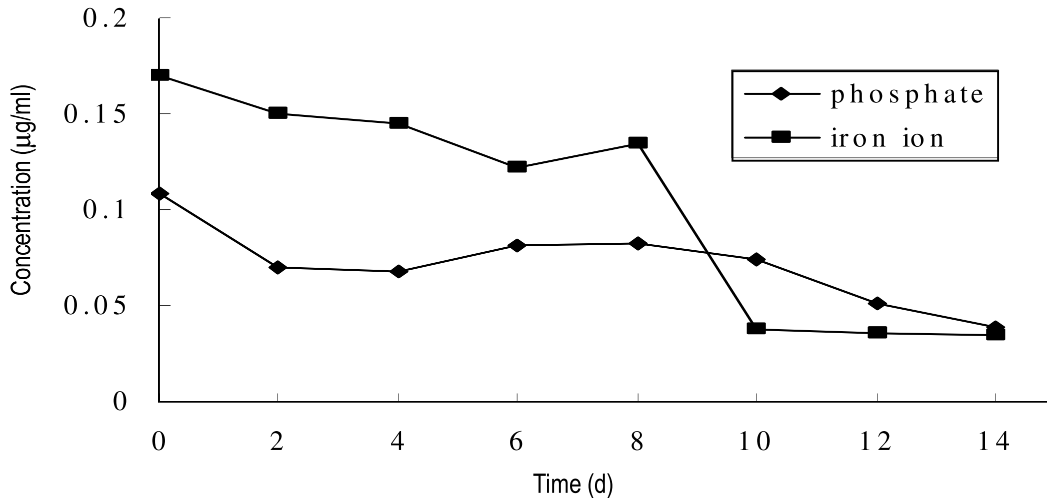

Fig. 6: Time course of phosphate and iron ion concentration in bioreactor culture medium of *P. lobata*

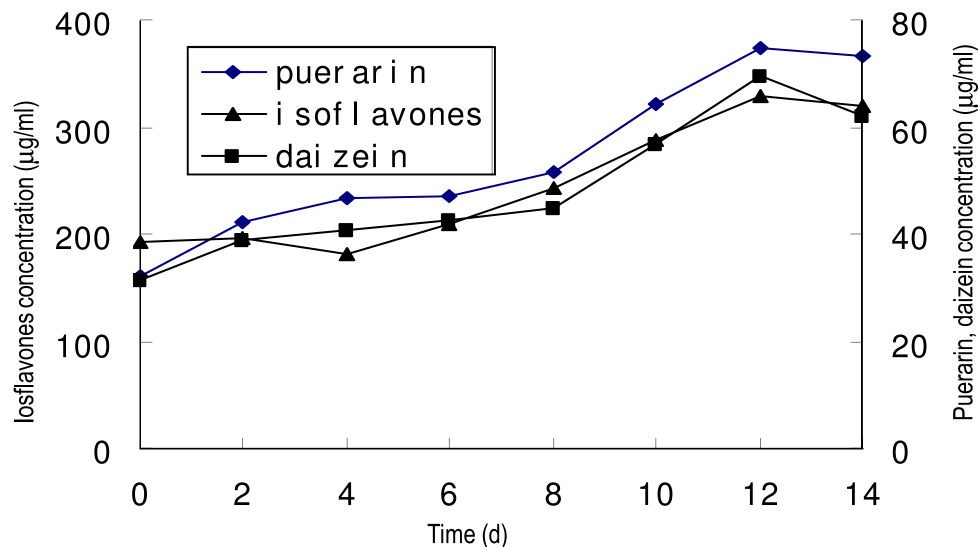

Fig. 7: Isoflavones, puerarin and daidzein accumulation in bioreactor culture medium of *P. lobata*

uptake of both ammonium and nitrate nitrogen started at the initiation of culture. After 14 days the uptake of ammonium and nitrate reached a similar rate (Fig.5). Only 50% of ammonium nitrogen was depleted over during the course of the culture. This suggests that the nitrogen concentration could not be utilized sufficiently. Liu and Zhong (1997) found that ginseng cell culture showed the same pattern of utilizing nitrogen sources.

During cell multiplication the phosphate concentration declined in the bioreactor culture of *P. lobata* (Fig.6). During the 2 day lag phase (d 1 to d 2), 50% of phosphate was utilized. A possible explanation for this strong absorption pattern is that cells were phosphorus deprived by the end of the suspension culture phase and compensated by absorbing relatively high amounts of phosphate for cell growth and reproduction when transferred to the new medium (Guo *et al*., 2003). During the latter half of the exponential phase (d 8 to d 12) the concentration of phosphate declined slowly. Even in the stationary phase (d 12 to d 14), phosphorus was absorbed by cells (Fig. 6). This suggests that cells could store phosphorus for cell growth and biosynthesis and is consistent with the proposal above of phosphorus deprivation and accumulation. The phosphate was depleted about 70% at the end of the run (Fig. 6).

Iron is an essential element for plant growth and development (Fan *et al*., 2004). In our bioreactor study, Fe^{2+} was gradually extracted from the medium during the lag phase. However, the consumption of Fe^{2+} rapidly increased during the latter part of the exponential phase. Total iron ion was exhausted about 80% after 14 days (Fig.6).

In our results, nutrient exhaustion varied with different cell growth stages. At the initial, slow cell growth phase, little nutrient uptake occurred. During the exponential growth phase, there was a linear relationship between nutrient element exhaustion and cell growth. At the stationary phase, nutrient exhaustion was almost complete.

Accumulation of total isoflavones, puerarin and daidzein in bioreactor medium: Extracellular accumulation of isoflavones can be seen from Fig. 7. There was little isoflavone production during the initial 4 days, which then increased from d 4 to d 12, indicating that the process was strongly coupled with cell growth in *P. lobata* culture. From day 2, during the exponential growth phase, puerarin and daidzein increased slowly. The extracellular puerarin and daidzein production peaked at d 12. This phenomenon was also found in airlift bioreactor culture of *Pueraria phaseoloides* (Kintzios *et al.*, 2004). In shake flask culture, the production of puerarin from *P. lobata* was strongly coupled with cell growth and puerarin yield peaked on the 8th day, whilst total isoflavone yield peaked on the 12th day (Zhang and Li, 2003a,b). This contrasted with our bioreactor culture in which both isoflavone and puerarin peaked on the 12th day. In airlift bioreactor culture of *P. Phaseoloides*, puerarin accumulation was almost 200 times as much as in 250 ml flask cultures (Kintzios *et al.*, 2004).

The results of this study showed that bioreactor culture of *P. lobata* can be used to produce isoflavones, puerarin and daidzein. Analysis of the accumulation patterns of puerarin and isoflavones in relation to nutrient consumption improved understanding of both the culture process and regulation of metabolism. It also demonstrated that bioreactor culture of *P. lobata* is probably a superior industrial production method for puerarin.

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