Introduction

Biosensor and bioreporter as indicator of chemistry pollution has been widely applied in many fields. But traditional bioreporters are not practical when it comes to in field application. One crucial factor that restricts the in field application of bioreporter is the preservation condition of the bacteria. It is quite impractical to cultivate certain strain of genetically engineered bacteria to achieve harvest in the field. So the best solution is the optimization of preservation condition, which can be applied in the field easily and quickly. We are trying to fulfill two goals: one is to preserve the bacteria in a powder for or a glassy form at non-costly condition, the other is to make the bacteria able to be recovered as quickly as possible and maintain a relatively high activity. By this way, bacteria reporters would be quite available in the field.

There are some alternatives to choose from, among which are the germination of spores to vegetative cells, low temperature preservation (-20 ℃), and freeze dried method. Unfortunately, all of them are not available, due to the low survival rate, or the long time to recover. It is now supposed that the most effective way is to ambient dry with a preservation media. Previous work indicated that a kind of sugar, trehalose, stabilizes membranes and proteins in the dry state, most likely by hydrogen bonding to polar residues in the dry macromolecular assemblages. This direct interaction results in maintenance of dry proteins and membranes in a physical state similar to that seen in the presence of excess water. Trehalose has an advantage over other kinds of sugars for it has a relatively high glass transition, so the bacteria can maintain a glassy form when temperature and humidity varies. It is also important to produce in cell trehalose by the bacteria spontaneously, which involves certain gene expression and an osmotic press induction. In *E. coli*, the *otsA* and *otsB* genes are responsible for trehalose biosynthesis from UDP glucose. These genes encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively. And high concentration of NaCl solution induces the expression of intracellular trehalose.

So the protection media should contain trehalose at least. And previous works also indicated that high concentration of polyvinylpyrrolidone (PVP) contribute to a relatively long time of maintenance. Previous work also suggested that adding an ounce of two kind of salt, sodium ascorbate and sodium glutamate, may have an effect on the preservation result. Our main task is to see whether these two salts are able to improve the effect of preservation, and to determine if a best result can be achieved by a higher concentration of trehalose!

Method

We prepared 4 types of protection media (Table-1).

	PVP	Trehalose	Sodium Ascorbate	Sodium glutamate
PT34(+)	150g	34g	2g	2g
PT34(-)	150g	34g	-	-
PT68(+)	150g	68g	2g	2g

PT68(-)	150g	6g	-	-

In sum, we constructed two strains of E.coli bacteria, BL21 bearing standard plasmid with T7 promoter + BBa_E0840 as an insert, which will express GFP when induced by IPTG; the other strain was developed from DH5 alpha, bearing plasmid with Constitutive Promoter+MerR gene and PmerT Promoter+BBa_E0840, which will express GFP when induced by Hg (II).

We firstly cultivated single colonies of each strain for 8 hours at 37 ℃. Then resuspend in 5 ml LB medium containing 2% NaCl for another 8 hours (for preconditioning under osmotic stress, and the bacteria in the NaCl media grows too slow). The cells were harvested by centrifugation (6 min at 3,800 ×g). Preliminary tests had indicated better survival resistance of stationary phase cells compared to cells from the exponential growth phase. Bioreporter preservation by vacuum drying stationary phase bioreporter bacteria (salt-preconditioned or not) were resuspended in different drying protection media to achieve culture optical density at 10. Here, we use four kinds of protection media. Ten microliters of the cell suspensions in protection media was applied to an Eppendorf tube and subsequently vacuum-dried at ambient temperature using water pump for about 10h. The resulted samples were glassy like rather than powder like, stored at 4 ℃.

We applied two methods to evaluate how the samples recover.

1. Colony-forming units and determination of total cell counts for determination of colony-forming units (CFU). We added 1ml of ddH2O on the sample to recover for about 30min in 37 ℃, and then added 100ul of the media to 900ul ddH2O, followed by applying 100ul of the dilution plated on LB agar plates. The plates were incubated overnight at 37 ℃ before counting the number of colonies.

2. We applied 1ml of LB media with inducer (1uM IPTG or 10^-7 uM Hg) to each sample for recovery, cultivating for about 10h. Then the cells were harvested by centrifugation (13000* 5min), and resuspended with 500ul PBS. Then GFP intensity was recorded by Microplate Reader, every hour to monitor the recovery situation. We also test the GFP intensity under different density of inducers to draw a dose response curve. We supplied LB media with different concentration of inducers (for IPTG from 10^-3 to 10^-7 and for Hg from 10^-5 to 10^-9), and cultivate for about 15 to 25 hours. For comparison, the same procedure was performed every time with fresh cells from the stationary phase of an overnight culture. Cell activities were characterized by the time until the color development started (referred to as the response time (RT)), the maximum corrected A600 achieved (the response intensity, RI).

Results

Short term preservation (20 days):

To identify the best preservation conditions for the bioreporter activity maintaining, we used 10^{-7} uM Hg (II) or 10^{-3} uM IPTG to induce the cells that had been preserved in different protocols. Fresh cells harvested in the stationary phase on each sampling day served as control.

After 20 days storage at 4° C, the samples were then recovered by incubating for 20h in LB media at 37° C. Figure 1-1 shows the OD values of the samples. The overall growth situation of samples in all different protection media was similar, while the T7p-GFP strains were more vigorous. The difference between the two trains more likely resulted from the inherent differences of the cells, rather than the effects of different protection media. So it can be concluded that the four different media have similar effects on the cells considering the growth situation. Figure 1-1 shows that the OD values of cells with or without inducers are approximately the same. So the inducers had little to do with the growth conditions, in other words, such concentration of inducer neither represses nor facilitates the growth of the cells.

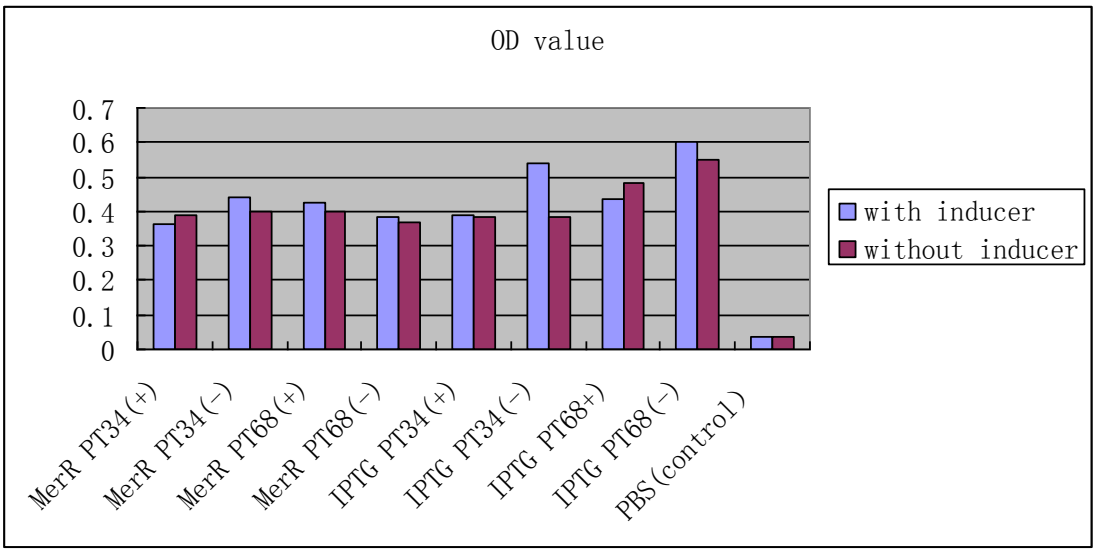

Figure 1-1 The samples were incubated in LB with/without inducer for about 10 hours, and then measured the OD value of each sample to compare the difference of growth situation with or without inducer

Figure 1-2 shows the response intensity of the samples, the control group was samples without inducer supplied. The T7p-GFP strain with PT68(-) media failed to express GFP. For the rest of the samples, the T7p-GFP strain had a much higher expression level of GFP than MerR-GFP strain. For T7p-GFP strain, sample of PT34(-) had the highest intensity, followed by PT68(+) and PT34(+). For MerR-GFP strain, the difference of expression level between samples with inducer and the control groups is slight, so it is hard to compare. Depending on the expression level of T7p-GFP strain, PT34(+) protection media has the best effect, while PT68(-) has the worst effect for cell protection. From Figure 1-2, the highest intensity of every sample was also indentified (see Table 1).

Figure 1-3 shows the value of GFP/OD, representing similar overall trend with Figure 1-2.

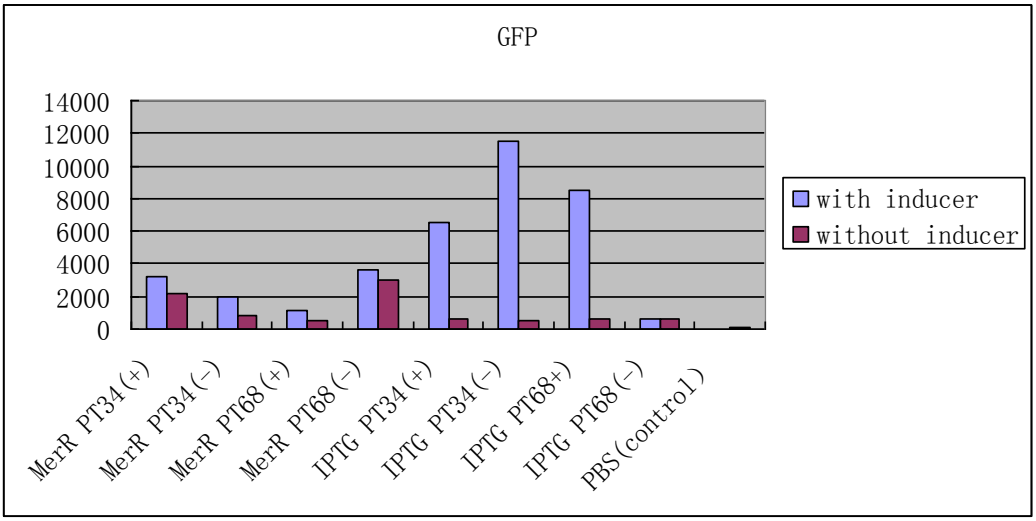

Figure 1-2 *The samples were incubated in LB with/without inducer for about 10 hours, and then measured the GFP intensity of each sample to compare the difference of growth situation with or without inducer*

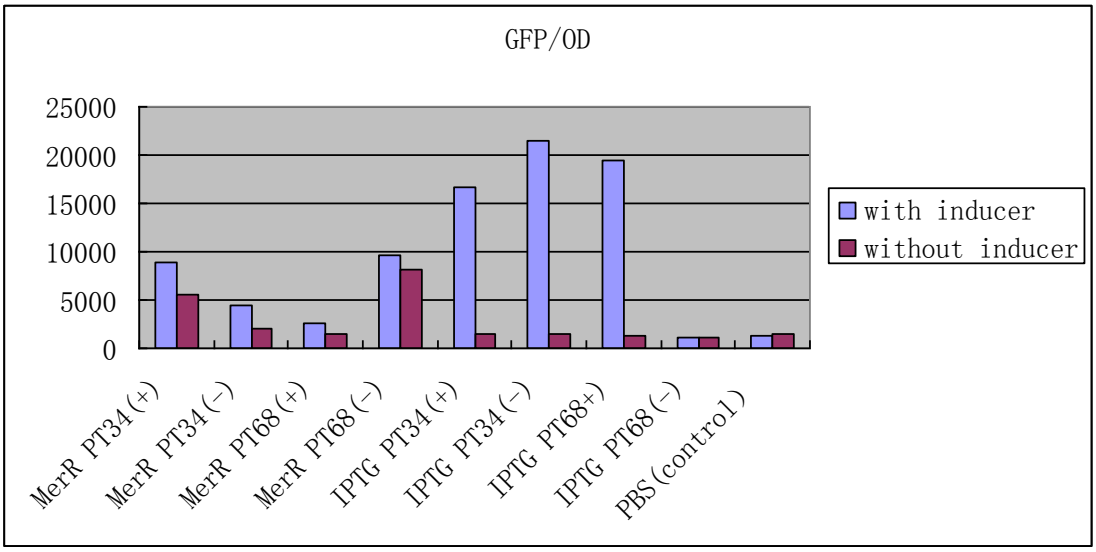

Figure 1-3 *With OD value and GFP above, we could determine the GFP/OD value of each sample.*

Figure 2-1 shows variations of all the samples OD value with incubation time. The OD values were measured after 7-10 hours. For MerR-GFP strain, the sample PT34(-) already began to grow after 7 hours, according to the OD values, approximately 2 in 7 hours and remaining the same for the next three hours , so their lag time esd actually shorter than 7hours. The lag time for PT34(+) and PT68(+) were 8 hours, while the lag time for PT68(-) was about 9 hours. For T7p-GFP strain, the sample PT34(-) remained a very low OD value through 7 to 10 hours, so it's lag time would expect to be well longer than 10 hours. Other samples' OD values gradually increased to about 0.05 to 0.2 or 0.25.

Figure 2-2 shows the variation of GFP intensity with incubation time. The T7p-GFP strain's expression level remained very low through the whole process. Their response times were longer

than 10 hours. For MerR-GFP strain, intensity of PT34 (-) and PT68 (+) were already very high in 7 hours, so their response were shorter than 7 hours. The response time of sample PT34 (+) was 8 hours and that of PT68 (-) time was 8 hours. Figure 2-3 shows the variation of GFP/OD value with time, and it reflected a similar results.

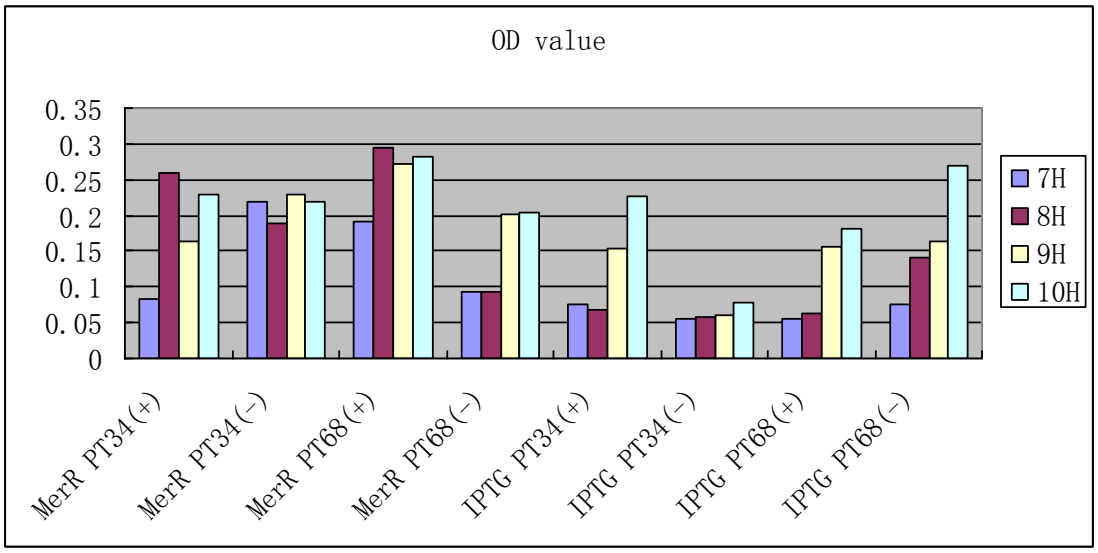

Figure 2-1 *We cultivated 12 samples composed of each strain and each protection media(MerR PT34+MerR PT34- MerR PT68+ MerR PT68- IPTG PT34+ IPTG PT34- IPTG PT68+ IPTG PT68-). Then record the OD value using 96-well plate. The OD value of each time point was the average of all three replicates.*

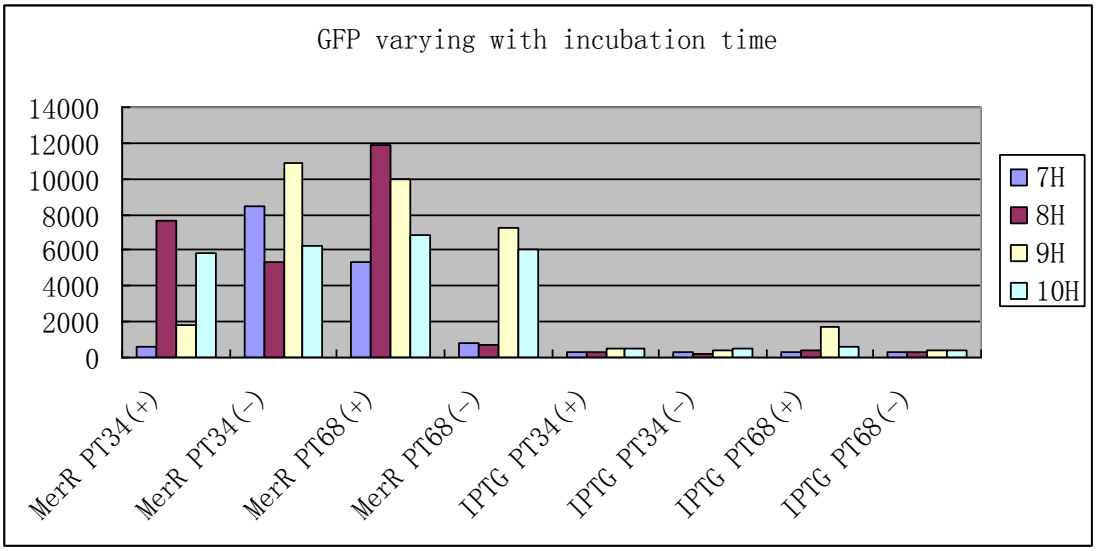

Figure 2-2 *We cultivated 12 samples composed of each strain and each protection media(MerR PT34+MerR PT34- MerR PT68+ MerR PT68- IPTG PT34+ IPTG PT34- IPTG PT68+ IPTG PT68-) After 7 hours, we harvested cells and protection media and then resuspended in PBS. Then pipette sample into 96-well plate for GFP detection. The GFP value of each time point is the average of all three replicates.*

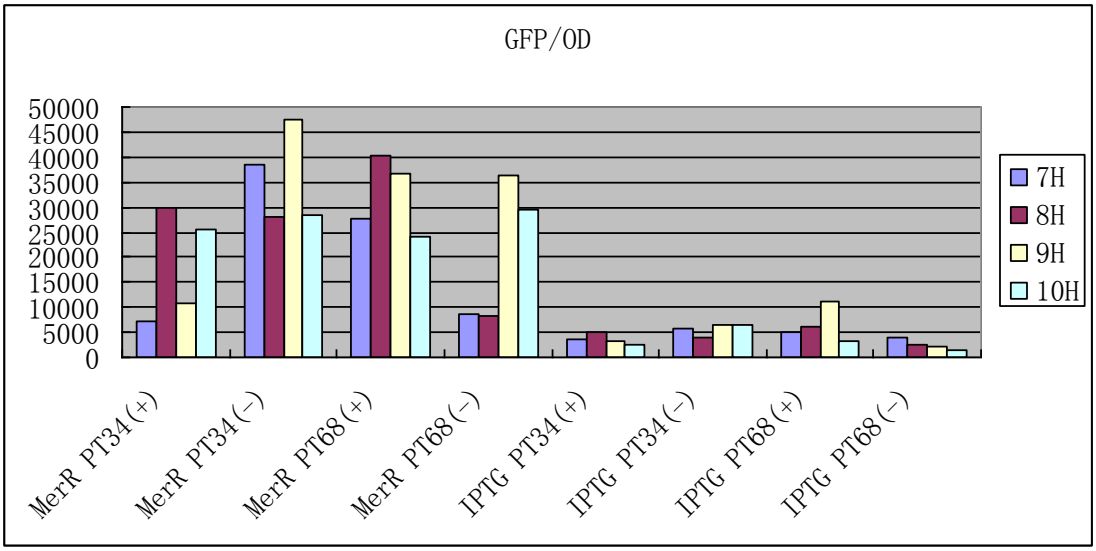

Figure 2-3 the *GFP/OD values were derived from the previous figures.*

To sum up the overall effects of all four kinds of protection media (Table 2), the differences between LT and RT are not considerable, but the HI varied widely from sample to sample. So according to this table of a summary data of samples storage for 20 days, suggesting that PT34 (-) has the best effort for protection of cells, while other kinds of protection media also had good effort for short term preservation.

Table 2 *lag time, response time and highest intensity of different samples*

	PT34(+)		PT34(-)		PT68(+)		PT68(-)	
	MerR	T7P	MerR	T7P	MerR	T7P	MerR	T7P
LT	8	9	<7	>10	8	9	9	7
RT	8	>10	<7	>10	<7	>10	9	>10
HI	3000	6500	2000	11000	1000	8500	3500	500

Long term preservation: (50 days)

After 50 days' storage at 4° C, the overall condition of samples changed compared with that of 20 days. Moreover, due to the long preservation time, even the situation of samples of identical protection media can vary widely, some of which still contained considerable number of active cells, some of which, however, hardly had any viable cells. So it is very likely that the samples cultivated for 24 hours contain few active cells, while the samples cultivated for 20 hours contain much more active cells. So in the OD value of samples cultivated for longer time was lower than that of shorter time (Figure 3-1). Thus, it is hard to predict the lag time of each sample. The same thing happened for the data of GFP value detection (Figure 3-2). But the GFP expression of MerR PT34 (+) and T7p PT34 (+) remained in an extremely low level through 20 to 24 hours. The GFP expression level of sample MerR PT34 (-) MerR PT68 (+) T7p PT68 (+) T7p PT68 (-) reached a very high level. to conclude, the protection media PT68 (+) has the best effect.

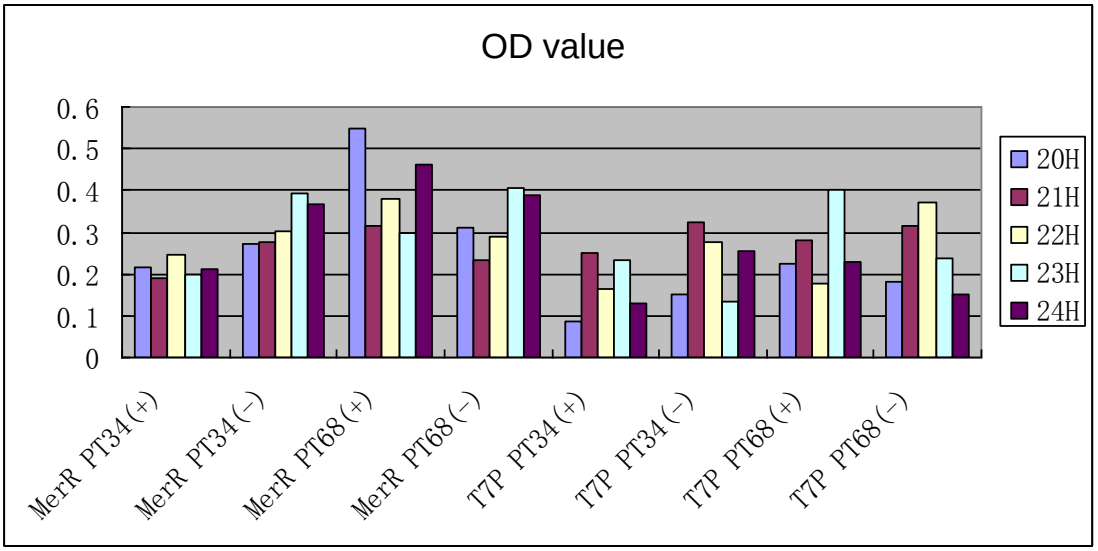

Figure 3-1 *We cultivated 12 samples composed each strain and each protection media(MerR PT34+MerR PT34- MerR PT68+ MerR PT68- IPTG PT34+ IPTG PT34- IPTG PT68+ IPTG PT68-) After20 hours, we harvested cells and then resuspended with PBS. The OD value recording was performed in 96-well plates. The OD value of each time point is the average of 3 replicates.*

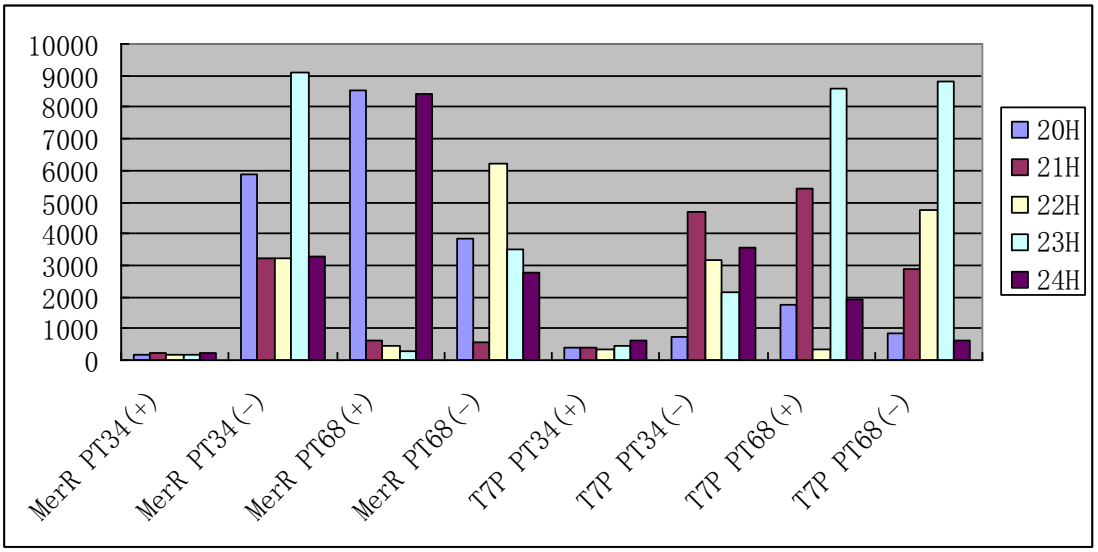

Figure 3-2 *We cultivated 12 samples composed each strain and each protection media (MerR PT34+MerR PT34- MerR PT68+ MerR PT68- IPTG PT34+ IPTG PT34- IPTG PT68+ IPTG PT68-) After20 hours, we harvested cells and then resuspended with PBS. The OD value recording was performed in 96-well plates. The OD value of each time point is the average of 3 replicates.*

Future work

There are many aspect of our work need to be further refined.

First, our water pump cannot supply a high degree of vacuum. Previous work has shown that a vacuity of less than 0.04 mbar can easily turn the sample into a powder form, while our samples

only achieved a glassy form. If we would have use a more powerful pump, I suppose that the effect would be much better, for the quantity of remaining water matters a lot. Second, our way to detect the OD value and GFP of the samples were not accurate enough. A real time detector which can maintain the growth of the samples, and meanwhile detect the data would be much useful to give more convincing figures.

On the other hand, the role of trehalose and salt in the protection media should be further studied. We have not sure whether 68g/L trehalose in the media is the best. To further determine that, media with a serial of concentration of trehalose should be made. What role does salt play in the media is also unknown.

More importantly, we noticed a phenomenon during the period of labs: after about 20 days' storage, many samples which protection media didn't contain Sodium ascorbate and Sodium glutamate turn from glassy form into icy form. But these samples still contain numerous viable cells. Why some samples turn to an icy form? How would it influent the preservation effect? We cannot answer these questions now.

All the problems and questions above need further study.

Reference:

Belkin S (2003) Microbial whole-cell sensing systems of environmental pollutants. Curr Opin Microbiol 6:206–212

Harms H, Wells MC, van der Meer JR (2006) Whole-cell living biosensors—are they ready for environmental application? Appl Microbiol Biotechnol 70:273–280

Tunnacliffe A, de Castro AG, Manzanera M (2001) Anhydrobiotic engineering of bacterial and mammalian cells: is intracellular trehalose sufficient? Cryobiology 43:124–132

Stabilization of Dry Membranes by Mixtures of Hydroxyethyl Starch and Glucose: The Role of Vitrification John H. Crowe, Ann E. Oliver, Folkert A. Hoekstra, and Lois M. Crowe
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