Caloric restriction causes symmetric cell division and delays aging in *Escherichia coli*

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Aging is one of the most intriguing processes of biology and despite decades of research, many aspects of aging are poorly understood. Aging is known to occur in bacteria and yeast that divide with morphological asymmetry[1, 2]. Morphologically symmetrically dividing bacteria such as *Escherichia coli* were assumed not to age until they were shown to divide with functional asymmetry leading to aging and death of some of the cells even in exponentially growing cultures[3]. In asymmetrically dividing *E. coli* the newly synthesized components are presumed to occupy one pole so that after division one of the daughter cells receives newly synthesized components whereas the other retains the older components[3, 4]. Mathematical models predicted that at the population level, asymmetric growth should result in higher growth rate[5, 6, 7] and symmetric growth in higher growth yield[7]. Therefore, arguably symmetric cell division should be selected in low nutrient environments and asymmetric division in nutrient rich environments. A further prediction was that lower substrate concentrations should strengthen repair mechanisms and suppress aging whereas higher substrate concentrations suppress repair and enhance aging[7]. We show here that *E. coli* divides

more symmetrically under caloric restriction, that both genetic selection and phenotypic plasticity are important determinants of cell division symmetry and also that the proportion of cells that stop dividing and therefore are presumably dead is significantly lower in symmetrically dividing cultures. However, contrary to the prediction, symmetry was not always accompanied by reduced growth rate. These results demonstrate that asymmetry of division in *E. coli* is not hardwired but responsive to the nutritional environment. This provides a new perspective on why caloric restriction increases lifespan in organisms ranging from microbes to mammals[8]. Symmetry of division may be a mechanism spanning across the width of life forms but regulating aging in different ways in different forms.

The central mechanism of cellular aging is likely to be cell division asymmetry with respect to the distribution of older and newly synthesized components. In animal systems asymmetric division is involved in differentiation and self renewing of stem cells and germline cells[9-12]. The phenomenon of asymmetry leading to aging can also be seen in unicellular forms such as bacteria and yeast. In budding yeast oxidatively damaged proteins remain preferentially in the mother cell whereas newly synthesized components occupy the bud cells[13] so that eventually the rate of reproduction of the mother cell declines until it stops dividing. In bacteria such as *Caulobacter sp.* that have a morphological asymmetry and differentiation, aging similar to budding yeast was demonstrated[2].

If asymmetric division is the key to cellular aging, unicellular organisms dividing symmetrically should be immune to aging as long as environmental conditions are favourable. It was shown however that fission yeast as well as *Escherichia coli* cells distribute the older and newly synthesized components to the daughter cells asymmetrically [3, 4, 14] resulting into one of the daughter cells being born older than the other. In fact, the two cells can be viewed as a parent cell and a daughter cell rather than as two sister cells. This

gives rise to a population dynamics that is similar to multicellular organisms that show age structured populations. Based on a Laslie Matrix model Watve *et al*[7] simulated symmetric and asymmetric division and its effects on population growth. Their model assumed that the efficiency of cellular components declined with age and growth rate of a cell was a function of the relative proportions of new and old components. In asymmetrically dividing cells although a proportion of cells accumulated older components and ultimately died, young cells were being continuously generated and therefore the growth rate of the population remained high. On the other hand, in a symmetrically dividing culture, all the cells retained a proportion of older components resulting in slower growth of the entire population. Other modelling approaches agreed on the growth rate advantage of asymmetric division[5, 6]. In the Leslie Matrix model, at an optimum rate of repair the growth yield or biomass conversion efficiency of symmetrically dividing cells was predicted to be higher than asymmetrically dividing ones. Based on the simulation results Watve *et al*[7] argued that symmetric division was a better strategy under low nutrient conditions when biomass conversion efficiency would be more critical. On the other hand under a nutritionally rich but highly competitive environment, asymmetrically dividing cells would gain a reproductive advantage. A shift in cell division strategy may be observable in ecological or evolutionary time. There is a suggestion that asymmetric division in yeast may not be hardwired but responsive to environmental conditions [13, 15], although this possibility has never been rigorously tested. If the cells have phenotypic plasticity, they may change cell division strategies in response to the environment in a short duration. Alternatively optimum cell division may evolve by prolonged selection in a given environment. Such a change should be observable in evolutionary time.

In order to test these predictions, we reared four strains of *E. coli*, starting with an environmental isolate that was able to grow on both nutrient rich and dilute media, on two

types of substrates, one with glucose as the sole source of carbon and energy and the other with a complex substrate - peptone, each in 1 and 0.01 g/dl concentration. In order to keep the temporal variation in substrate concentration to a minimum, the cultures were transferred by mid to late exponential phase so that during selection period none of the cultures faced starvation and stationary phase (we denote the strain selected under high caloric conditions as *H* and the one selected under low caloric conditions as *L*. The current medium condition for the experiment is denoted by small letters *h* and *l* for high and low caloric conditions respectively, e.g. a strain selected under high concentration but currently being grown in low concentration is denoted as *Hl*). After an estimated 500 and 1000 generations of selection each of the strains was examined for growth rate, growth yield and symmetry of cell division on both high and low nutrient media.

Consistent with the expectation, the strain selected under low glucose concentration gave higher yields expressed as total cell protein per unit substrate consumed as compared to the strain selected under high glucose concentration at any given current glucose concentration, i.e. growth yield of *Ll* was higher than that of *Hl* and *Lh* was higher than *Hh*. Selection in low or high concentration of peptone had similar effect when grown in glucose (figure 1) showing that the effect of selection was not substrate specific. Peptone being a complex substrate, growth yield could not be calculated precisely but *Lh* gave higher stationary phase absorbance than *Hh* in glucose as well as peptone media (data not shown). The growth of any of the strains was slower in dilute media but the effect of selection on growth rate was complex. For *Lh* in peptone, the growth rate substantially declined by 500 generations as the growth yields improved. However, after 1000 generations of selection, growth rate was observed to be high and comparable to *Hh* in peptone. The strains selected in glucose, did not differ from each other in growth rates after 500 or 1000 generations although growth of both was faster than the ancestral strain.

For quantifying functional symmetry in cell division we defined an index of asymmetry based on the assumption that if the cell division was asymmetric, the daughter cell receiving older components will take longer to complete the next cell division[5]. Therefore an index of asymmetry was defined as the ratio of the difference in division time (sign ignored) of two sister cells as to the average division time of the two. Cell division observations were made by spreading mid exponential phase broth culture on to a slide layered with agar having the same nutrient composition as the broth. If the cell division in the broth culture was asymmetric, some of the cells transferred on the slide would have been old and therefore fail to grow or grow slowly. Therefore death and aging during the broth phase could be detected by failure or delay in the development of microcolonies. Subsequent divisions on the slide could be directly observed for about four generations. For each strain the mean index of cell division asymmetry was calculated after selection for approximately 500 and 1000 generations. According to the observations of Stewart *et al*[3] and the Watve *et al* model[7], asymmetry between two daughter cells grows as the cell ages. However in an exponentially growing culture the majority of cells are in the youngest age class that should show minimum asymmetry. As a result the frequency distribution of the index of asymmetry should always be positively skewed. The expected skewness was observed in all populations (see supplementary material). It can be argued that the cell division asymmetry could be a stochastic event and even chance asymmetries in cell size or component distribution will lead to an apparently positively skewed distribution. We tested this by fitting two alternative models to the distributions. Stochastic asymmetry around a mean of zero asymmetry will give a half-normal or mod-normal distribution, whereas accumulating asymmetry as in the Watve *et al* model[7] would give a negative exponential. Out of the six distributions namely those of *Ll*, *Lh*, *Hl*, *Hh* after 500 generations in glucose and the wild type in high and low calorie glucose (*Wh* and *Wl* respectively), normal distribution was rejected for all the six whereas

negative exponential was rejected only for one (see supplementary material) indicating that the asymmetry was accumulating as per the aging model and not stochastic.

Both, current substrate concentration and concentration during selection affected the cell division symmetry (Figure 2a). For wild type strain as well for strains *H* and *L*, for 500 or 1000 generations in glucose, the asymmetry index was significantly higher for *Hh* and *Lh* as compared to *Ll* and *Hl*. However after 1000 generations of selection, the *L* strains retained their symmetry in spite of current substrate concentration and the *H* strains retained asymmetry even in dilute environments. Thus prolonged selection seems to have resulted into commitment to symmetric or asymmetric division. In complex media the effect of current concentration was not significant whereas at both current concentrations strains selected under high concentration had significantly higher asymmetry (Figure 2b). In both the media and durations of selection, *Hh* showed significantly higher indices of asymmetry as compared to *Ll*. In multifactorial analysis the effect of selection on asymmetry index was highly significant and the effect of current concentration was marginally significant where as duration of selection and type of substrate were non-significant (see supplementary material for details of statistical analysis). Also selection under high concentrations resulted in significantly greater variance in asymmetry as compared to selection in low concentration. The results demonstrate that, both phenotypic plasticity and genetic selection determined the cell division strategy but prolonged selection resulted into loss of phenotypic plasticity suggesting that there may be a cost associated with plasticity that exerted a negative selection effect when plasticity was no more required.

The shift in the cell division symmetry in response to the substrate concentration can be a passive effect of reduced growth rate. It can be hypothesized that when growth rate is slow it may be difficult to maintain asymmetry owing to diffusional mixing of old and new components. As opposed to the model[7] the observed symmetry may only be an effect rather

than the cause of slow growth. To test this we used a vitamin B12 auxotroph of *E. coli* (*E. coli* 113-3D ATCC) and subjected it to similar selection under high and low glucose concentration for 500 generations. Because of its auxotrophic nature its growth rate could be regulated by the growth factor concentration and made independent of the energy source. Under growth factor limited conditions the index of asymmetry was significantly higher in the high concentration selected strain (median=0.19718) as compared low concentration selected one (median=0.01212) (Mann-Whitney U test; n1=58, n2=53, W=4272.5 and $p<0.0001$). This suggests that the concentration of the caloric nutrient influenced the symmetry of division rather than the growth rate itself or the concentration of the growth limiting nutrient.

To monitor possible death of cells we scanned between 500 to 700 developing microcolonies growing on each of the slide cultures that were prepared for cell division observations. The cells that failed to divide and form microcolonies when the modal microcolony size exceeded 16 cells were taken as presumably dead during the broth phase of growth. The proportion of such 'left behind' cells was higher when cells were exposed to different caloric environment than what they were selected for (i.e. *Hl* and *Lh*). When the selection and current caloric conditions were similar (*Hh* and *Ll*), there was a significant positive correlation between cell division asymmetry and the proportion of left-behind cells (Figure 3). This is compatible with the theoretical prediction that asymmetric division leads to aging whereas symmetric division should protect from aging. Further there was a negative correlation between asymmetry and growth yield (Figure 4). The observations support the predictions of the Watve *et al*[7] model that strains adapted to low nutrient concentrations should adopt symmetric division, show a lower rate of aging and presumable death and have a higher growth yield. The results differ from the model prediction in that symmetric division did not obligately reduce growth rate. It is particularly interesting to note that in the strain *L*

on peptone, there was a substantial reduction in growth rate after 500 generations but a rise again by the 1000th generation. This suggests that adopting symmetric cell division may have reduced the growth rate initially but it may have been compensated later by positive selection on other mechanisms determining growth rates. We routinely subcultured exponential phase cultures to avoid starvation or caloric fluctuations during the experimental evolution. This could have selected for higher growth rates in all the strains irrespective of substrate concentration.

It is well known that caloric restriction leads to longevity in widely differing organisms including yeast, *C. elegans, Drosophila,* and mammals[8]. We demonstrate here that a similar phenomenon occurs in *E. coli* and operates by modulating the symmetry of cell division. Caloric restriction causing symmetric cell division could be a more general phenomenon not restricted to *E. coli.* In low calorie environments fission yeast showed synchronous cell division cycles[16]. Although Cheng *et al*[16] did not specifically quantify symmetry of division, maintenance of long term synchrony is not possible without cell division symmetry. It is possible therefore that caloric restriction resulted in symmetric division in yeast in their experiments. Further evidence for an association between caloric restriction, symmetry and aging in yeast is that mutation in Sir2, a gene necessary for asymmetric segregation in yeast[13], resulted in a longer chronological life span under caloric restriction[17]. It appears that Sir2 activation, leading to asymmetric segregation, reduces the chronological life span in yeast[17, 18] although its role in reproductive life span extension is debated[18, 19].

In unicellular organisms asymmetric division may have evolved to increase the growth rate of the population by continually generating young cells, where as in multicellular organisms asymmetric division seems to rejuvenate stem cells[9-12]. This creates an apparent contradiction. Symmetric division seems to delay aging in unicellular organisms as we show

here whereas asymmetric division is crucial for maintenance of stem cells and thereby delaying aging in complex organisms. If caloric restriction induced symmetric division in all types of organisms, its effects should be opposite in unicellular and multicellular organisms. However, there is another possible effect of caloric restriction that the model predicted which may explain its almost universal effect on longevity. Symmetric cell division should be accompanied by the optimization of repair rates according to the Watve et al[7] model. Our experiments could not test this prediction directly. However, in the model, increase in the growth yield accompanying symmetric cell division critically depended on optimization of repair rates. Therefore higher growth yield can be taken as an indirect evidence for higher repair rates accompanying symmetric cell division. Yeast grown under caloric restriction had lower mutation rates[16] suggesting that the triangular relationship between caloric restriction, cell division symmetry and repair rates could be more generally true and responsible for longevity in widely differing organisms.

Our experiment involving division symmetry in a growth factor limited auxotroph suggests that the available caloric nutrient, rather than the actual utilization of the nutrient, influenced cell division symmetry and aging. This is an interesting parallel to the finding in *Drosophila* that perception of food rather than actual intake of food influenced longevity[20]. Owing to the parallels as well as important differences in the aging processes of bacteria and higher organisms, it looks possible now that the inclusion of *E. coli* among the model organisms for aging research may throw light on some of the yet undiscovered aspects of aging.

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Footnotes to figures:

Figure 1: The effect of selection in high and low caloric environment on the growth rates ($hour^{-1)}$ and growth yields (mg protein per mg glucose consumed). Strains were selected in glucose or peptone media but determination of growth rates and growth yields was made in glucose alone since growth yield calculation in complex substrate cannot be precise. Strains L showed significantly higher yields as opposed to the strains H (paired t test, n=8, t= 7.2 p, two tailed = 0.000177). There was no significant difference in growth rates (paired t test, n=8, t=1.6979, p, two tailed= 0.1333). The correlation between growth rate and growth yield was negative as expected but not significant ($r = -0.3992$, $p > 0.05$).

Figure legends: Black triangles: The wild type environmental isolate

Blue Squares: Strains *L* after 500 generations.

Blue Circles: Strains *L* after 1000 generations.

Orange Squares: Strains *H* after 500 generations.

Orange Circles: Strains *H* after 1000 generations.

Figure 2: The median index of cell division asymmetry as influenced by current and selection substrate concentration. (Colour codes and shapes same as in figure 1). Asterisks on the right indicate significance for differences due to current substrate concentration for a given strain in pair-wise comparisons using Mann-Whitney test, those above or below the points indicate significance of difference due to selection on a given current concentration. (see supplementary material for detailed statistics) a) Selection and growth on glucose medium: The current glucose concentration affected symmetry in the wild type and in the 500 generation selected strain but after 1000 generations of selection the cells appeared to be committed to symmetric or asymmetric growth and did not respond to current concentrations. b) Selection and growth on complex medium: Trends on complex medium were similar to

that of glucose but there was less of asymmetry overall and the effect of current concentration was not significant.

Figure 3: Cell division asymmetry and proportion of presumably dead cells. ($n=12$, $r = 0.6083$, $p<0.05$) (Colour codes as in Figure 1)

Figure 4: Cell division asymmetry and growth yield. ($r = -0.6266$, $p < 0.05$) For all the experimentally evolved strains substrate concentrations during selection and observation were the same. (Colour codes as in Figure 1)

Figure 1

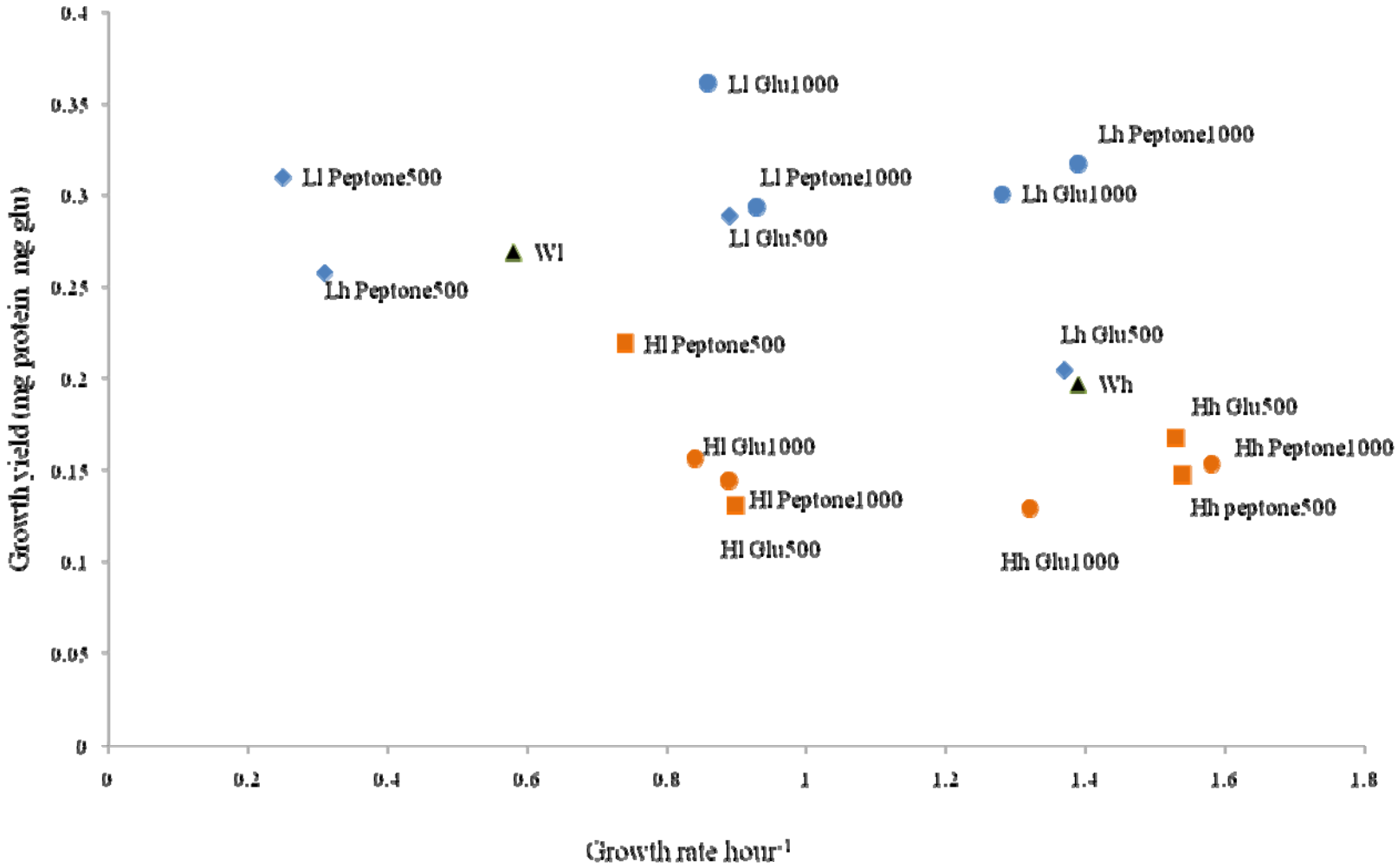

Growth yield (mg protein mg glu)
Growth rate hour-1
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
Ll Glu1000
Lh Peptone1000
Ll Peptone500
Ll Peptone1000
Lh Glu1000
Ll Glu500
Wl
Lh Peptone500
Hl Peptone500
Lh Glu500
Wh
Hh Glu500
Hl Glu1000
Hh Peptone1000
Hl Peptone1000
Hh peptone500
Hl Glu500
Hh Glu1000

Figure 2

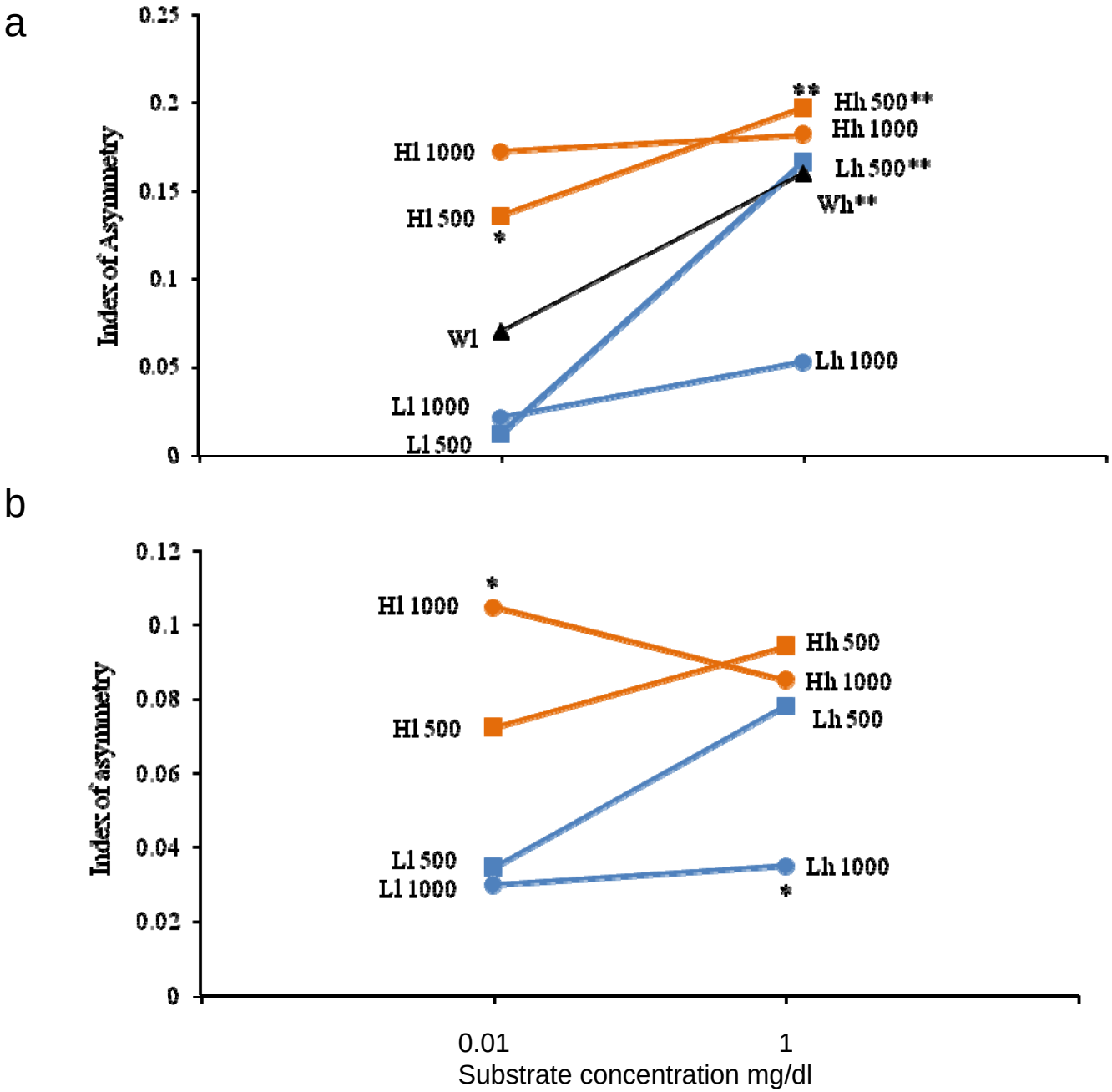

a
Index of Asymmetry
0.25
0.2
0.15
0.1
0.05
0
Hl 1000
Hl 500
*
**
Hh 500**
Hh 1000
Lh 500**
Wh**
Wl
Lh 1000
Ll 1000
Ll 500
b
Index of asymmetry
0.12
0.1
0.08
0.06
0.04
0.02
0
Hl 1000
*
Hl 500
Hh 500
Hh 1000
Lh 500
Ll 500
Ll 1000
Lh 1000
*
0.01
1
Substrate concentration mg/dl

Figure 3

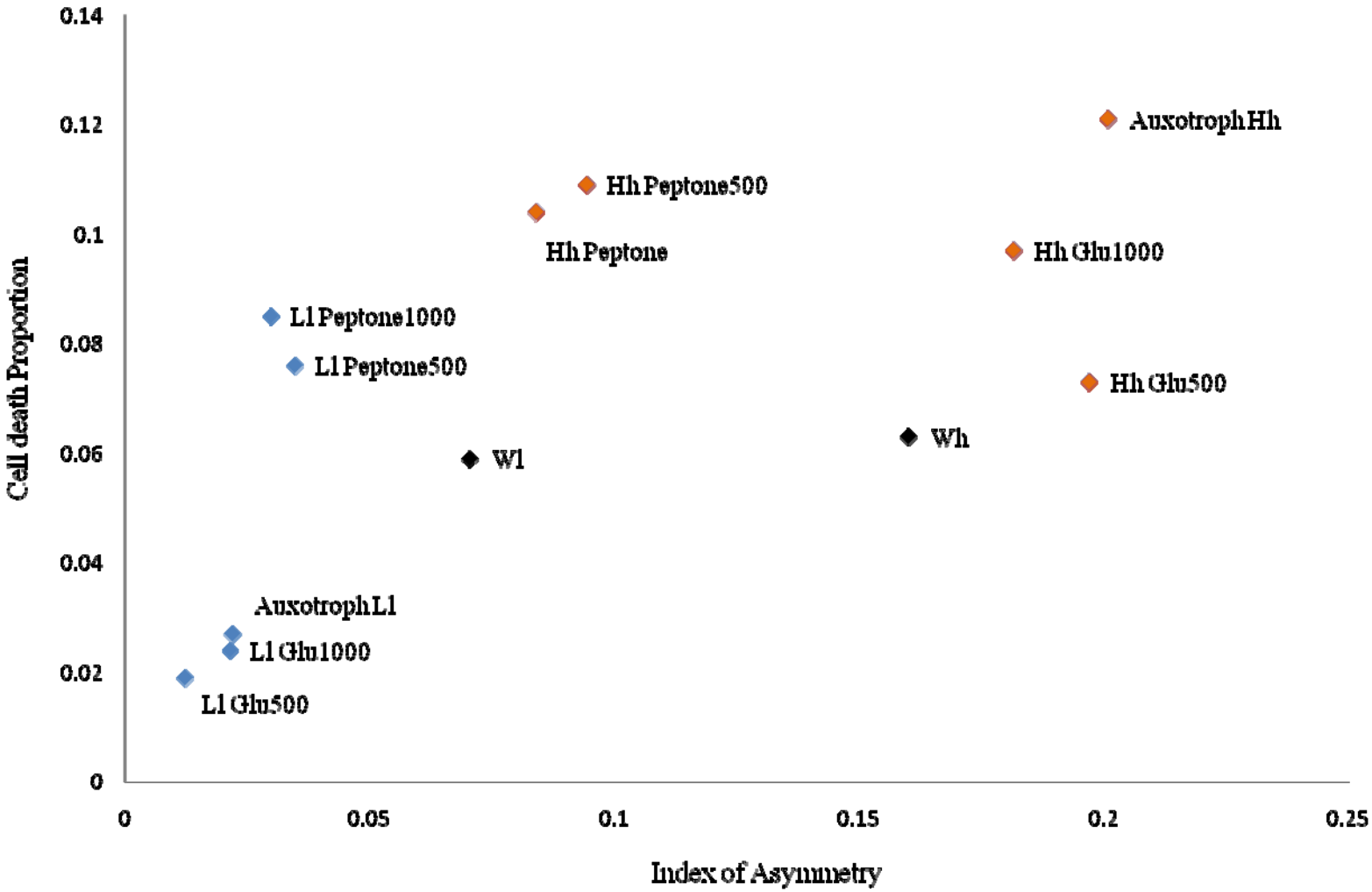
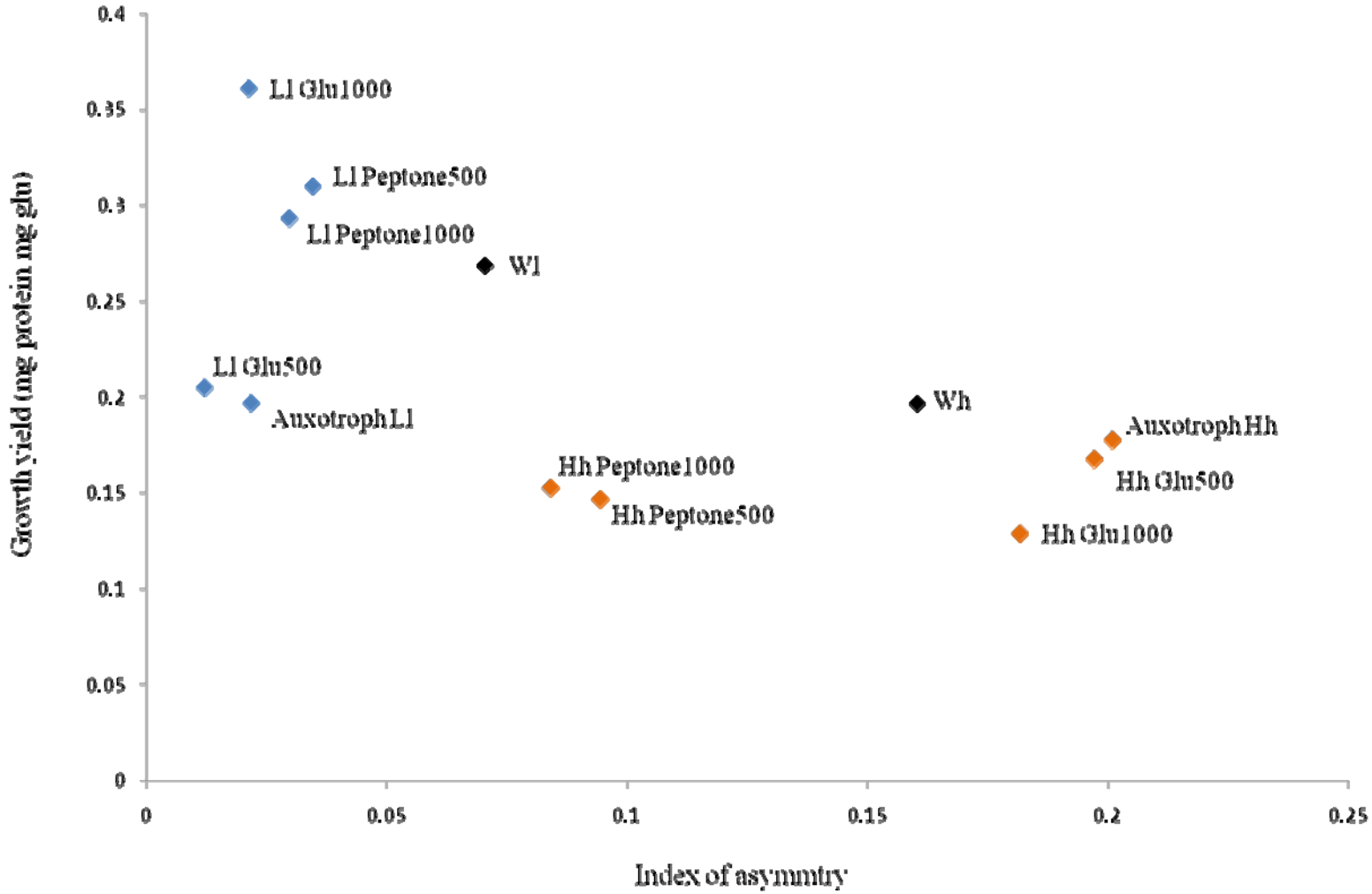

0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Cell death Proportion
0
0.05
0.1
0.15
0.2
0.25
Index of Asymmetry
Auxotroph Hh
Hh Peptone500
Hh Peptone
Hh Glu1000
L1 Peptone1000
L1 Peptone500
Hh Glu500
Wh
Wl
Auxotroph L1
L1 Glu1000
L1 Glu500

Figure 4


Growth yield (mg protein mg glu)
Index of asymmtry
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.05
0.1
0.15
0.2
0.25
Ll Glu1000
Ll Peptone500
Ll Peptone1000
Wl
Ll Glu500
Auxotroph Ll
Wh
Auxotroph Hh
Hh Peptone1000
Hh Glu500
Hh Peptone500
Hh Glu1000