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The Effects of Temperature on the Secretion of *S. pombe* Acid Phosphatase in the Yeast *Saccharomyces cerevisiae*

By

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ABSTRACT

The rate of secretion of *S. pombe* acid phosphatase from the yeast *Saccharomyces cerevisiae* is examined to determine the effect of temperature on the secretion of a foreign proteins. Yeast normally grows optimally at 30 °C. However, this may not be the optimal temperature for extracellular protein production.

In this report, a method for measuring the secretion rate of acid phosphatase (AP) is presented. p-nitrophenol phosphate (colorless) is reacted with AP to produce p-nitrophenol (yellow) and inorganic phosphate. Extent of reaction is measured by a spectrophotometer.

Studies on maximum specific growth rate in the temperature range of 20 to 40 °C show that the optimal temperature for growth is 35 °C; while the highest rate of secretion, both total, Vp, and extracellular, Vs, occurs at a significantly lower temperature of 25 °C.

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INTRODUCTION

In the last few years yeast has become increasingly more prominent in the pharmaceutical industry because it offers many advantages over other microorganisms. Yeast is unicellular, eucaryotic, and easy to grow in large-scale fermenters. Since it lacks endotoxins, yeast has been labeled Generally Recognized As Safe (GRAS) which is advantageous in the production of medical products for human consumption. In addition, yeast is genetically well-characterized and can be manipulated through recombinant DNA techniques to produce a diversity of human proteins such as human epidermal growth factor, interleukin-2, α-interferon, and tissue plasminogen activator [1,2]. Lastly, since yeast is eucaryotic, it possesses mechanisms for secretion which are similar to higher eucaryotes such as man.

The secretory aspects of yeast mentioned above have been the subject of considerable research for several reasons. First of all, unlike bacteria, yeast performs the necessary post-translational modifications of secreted proteins to make them fully active [3]. Next, secretion of fully active proteins is conducive to continuous fermentation, which offers advantages in economy and uniformity of product quality [4]. Finally, and most important economically, the inverse relationship between separation costs and final concentration of the product in the media provides an incentive towards maximizing the amount of protein secreted during the fermentation [5].

Many environmental factors can affect the efficiency of the yeast secretory pathway including Ca^{2+} concentration, tunicamycin concentration, and osmolality [6]. In particularly, the temperature of

the growth medium may significantly influence the rate of secretion of a foreign protein from yeast.

There are several possible explanations for this temperature dependence. First, temperature has an acute effect on yeast growth. At lower than optimal growth temperatures, cell metabolism is slowed down and enzyme activity is diminished [7]. These phenomenon may also affect secretion rate. Secondly, protein folding, an important component of secretion, is highly temperature dependent [8]. As the temperature rises, proteins becomes progressively more "unfolded." Heat-shock proteins bind to unfolded proteins in the endoplasmic reticulum (ER) preventing their secretion. Lastly, temperature alters the permeability of cellular membranes. At lower temperatures lipid bi-layer membranes become more rigid which may inhibit transport of proteins through the yeast secretory pathway.

Because temperature effects secretion in conflicting ways, it is speculated that an optimal temperature exists for protein secretion. This optimal secretion temperature may or may maybe not coincide with optimal growth temperature. By examining the rate of secretion of *S. pombe* acid phosphatase from the yeast *Saccharomyces cerevisiae* in the range of 20 to 40 °C, this paper will demonstrate that there exists an optimal temperature for secretion which is not the optimum temperature for growth.

MATERIALS AND METHODS

Yeast Strains and Medium SE7-6: pYE αFPHO (αFPHO) and SE7-6: pYE (pYE) were the two strains of *Saccharomyces cerevisiae* were used in this study. αFPHO contains a plasmid with an a-factor

promoter sequence and genes coding for the production of *S. pombe* acid phosphatase and tryptophan. To account for endogenous acid phosphatase production by SE7-6, all experiments were duplicated with a control strain, pYE, which contains a tryptophan gene on its plasmid. Since yeast does not normally produce tryptophan, the tryptophan gene on the plasmids of each strain allows for selection against those cells which lose the plasmid.

Culture Conditions The media used in all of the experiments, SD-CAA (recipe in Appendix A), contains no tryptophan and is selective for those strains which can produce their own tryptophan such as pYE and αFPHO. Based on experiments relating pH and growth rate (Figure 1 in Appendix C), the pH of the media was buffered at pH = 5.0 ([citrate] = 50 mM) which is a safe pH for yeast growth and an effective deterrent to bacterial growth.

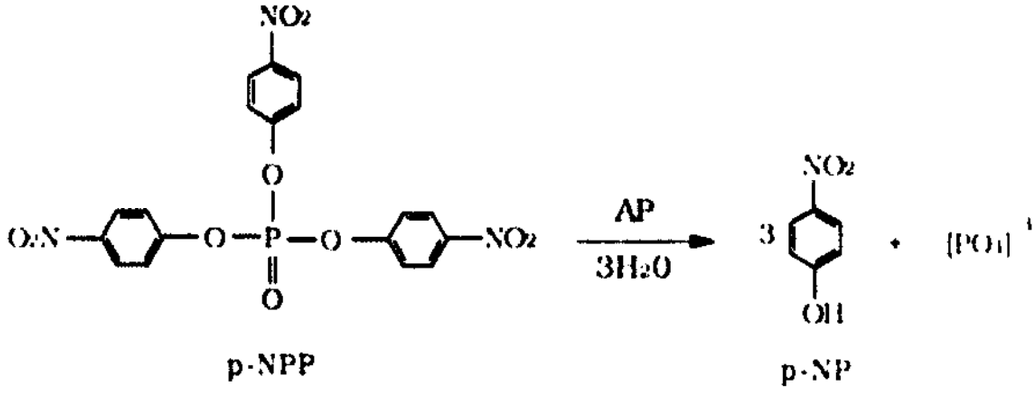
Strains were stored on agar slants of SD-CAA at 4 °C. SD-CAA plates of both pYE and αFPHO were streaked with culture from the slants and incubated at 30 °C for 3-4 days. For each strain, one overnight culture, consisting of 4 ml of liquid SD-CAA in a test tube, was inoculated with an isolated colony from a 3 day old plate. The overnights were grown at the experimental growth temperature for approximately 24 hr to acclimate the cells to the experimental conditions.

Experiments were performed in 250 ml side-armed erlenmeyer flasks. The flasks were inoculated with either 0.1 ml of pYE or 0.25 ml of αFPHO overnight and placed in a Magni-whirl constant temperature shaker bath. The lag time (time to reach exponential

growth) was about 9 hours for pYE and about 12-24 hours for αFPHO, depending on the growth conditions.

Growth Measurement A Klett meter was used to monitor growth during all experiments. This spectrophotometric method of measuring cell concentration was preferred over a dry cell mass assay because it is non-intrusive, aseptic, and more accurate at lower cell densities. The range of linearity between Klett reading and dry cell mass was determined by experimentation (See Figure 2 in Appendix C) to be between 25 and 100 Klett units which is the range of sampling for the enzyme assay. A detailed procedure is given in Appendix B for determining the maximum specific growth rate, μ_m, from Klett data.

Acid Phosphatase Assay Acid phosphatase (AP) is a non-selective enzyme which catalyzes the removal of phosphates under acid conditions. The assay used throughout this investigation involves the AP catalyzed cleavage of p-nitrophenol phosphate (p-NPP) to p-nitrophenol (p-NP) and inorganic phosphate:



This phosphate cleavage creates an aromatic product (p-NP) which turns yellow under alkaline conditions (deprotenation of the hydroxl). The amount of AP in the sample can then be measured indirectly by

measuring the absorbance of the reaction products at 435 nm. A detailed protocol for the assay is given in Appendix B and Figure 3 in Appendix C.

Plasmid Containing Fraction S. *pombe* acid phosphatase is very toxic to αFPHO which produces it constitutively. Therefore, there is a selection pressure against those cells which have the foreign AP gene. A previous co-worker found that αFPHO maintains its plasmid in only about 10% of the cell population, which means that all specific secretion rates for αFPHO presented in this paper are about an order of magnitude too low. A procedure for measuring the plasmid containing fraction of a cell population is given in Appendix B.

RESULTS AND DISCUSSION

Effect of Temperature on Growth Figures 4 in Appendix C shows the effect of temperature on the growth of αFPHO and pYE. The toxic effects of AP on αFPHO become clear when comparing its growth to pYE. In both cases the specific growth rate increases with temperature until a maximum of around 35 °C and drops off sharply above 35 °C. Throughout the range of temperatures tested in this report αFPHO grew much slower than pYE. In particular, at lower temperatures (<25 °C), αFPHO exhibits the phenotype of a cold-sensitive mutant. At 20 °C the growth rate was nearly zero for αFPHO but was about 0.14 (1/hr) for pYE. Most likely, the cold temperatures interfere with the normal protein secretory pathway by decreasing the permeability of the cell membranes.

Modifications to the AP Assay The procedure for the acid phosphatase assay, as outlined in Appendix B, is generally the same as the original assay developed by a previous co-worker. However, there have been a few changes which have made significant improvements in the reproducibility of the assay.

The assay initially suffered from inconsistent data and poor reproducibility. These problems were quite easily taken care of by switching to a larger sized cuvet (10 ml) in the spectrophotometer. The old "micro" cuvets provided only 4 ml for the width of the UV light beam. Because of the new, larger volume cuvets, three time as much saturated sodium carbonate was added to the reaction to quench it and provide alkaline conditions. This extra dilution improved the reproducibility of the highest secreting samples (Abs=0.4 0.6) since the Shimadzu in only linear for p-NP up to about 0.5.

Even with the extra dilution, the higher secreting samples still demonstrated a slow upward drift in absorbance with time which created artificially high secretion rates. The reason for this was that the cell positioner temperature was too warm at 30 C. By simply decreasing the temperature to 20 C, the reproducibilty of the most important samples was increased, and the hurried atmosphere of the assay was diminished.

Determination of Secretion Rates. One of the main objectives of this study was to decouple the growth rate from the secretion rate in this model system. In order to do this, mass balances must be written on AP attached to the cell wall and AP secreted into the medium:

$$\frac{APs}{dt} = Vp - Vs - \mu APs \qquad (1)$$

and

$$\frac{dAPo}{dt} = Vs\ N \qquad (2)$$

where APs = cell associated AP activity, APo = AP activity in the media, Vp = total rate of secretion of AP, Vs = rate of AP secretion into the media, N= cell concentration, and μ = specific growth rate ($dN/dt = \mu N$). Substituting for μ into the top equation gives

$$\frac{APs}{dt} = Vp - Vs - \frac{1}{N}\frac{dN}{dt} APs. \qquad (3)$$

Multiplying both sides by dt and integrating yields

$$\int_{AP(0)}^{AP(t)} dAPs = \int_{0}^{t} (Vp - Vs)\, dt \int_{N(0)}^{N(t)} \frac{APs}{N} dN. \qquad (4)$$

or simplifying the trivial first integral,

$$APs(t) - APs(0) + \int_{N(0)}^{N(t)} \frac{APs}{N} dN = \int_{0}^{t} (Vp - Vs)\, dt. \qquad (5)$$

By taking least squares fit of the cell-associated absorbance readings obtained from the spectrophotometer vs time one obtains the activities, APs(t) and APs(0), for each of the Klett readings (25, 50, 75, 100) taken during exponential phase. Plugging in these numbers into (5) and numerically integrating by the trapezoid rule, one obtains values for the right side of (5), which when plotted vs time, yield a straight line (if Vp and Vs are constant throughout exponential phase) with slope (Vp - Vs). This slope represents the rate of accumulation of AP between the cell wall and the cell membrane.

To obtain values for the total and extracellular secretion rates, Vp and Vs, one must integrate (2) above to get:

$$\int_{AP(0)}^{AP(t)} dAPo = \int_{0}^{t} Vs\, dt \qquad (6)$$

Again, plugging in activities (now from supernatant absorbance readings), using the trapezoid rule, and plotting the right side of (6) vs time gives a straight line with slope Vs. Vp is obtained by adding the slopes (Vp - Vs) and Vs.

Experimental Results The results of 22 experiments on secretion between 22.5 and 37.5 C are plotted in Figure 5. The total rate of acid phosphatase secretion, Vp, shows two significant peaks with respect to temperature. The first, at 25 C is almost an order of magnitude higher than the rate of secretion at 30 C. Similarly, the second peak, at 35 °C, is about 5 times higher than the secretion rate at 30 C. The secretion rate of AP into the media also shows a maximum near 25 C and a minimum near 30 C. The endogenous AP secretion, as measure by the secretion rates, Vp and Vs, for pYE, is insignificant, and ignoring it will have no effect on the conclusions drawn in this paper.

Literature Survey In general, very little attention has been given in the literature to the effects of temperature on secretion. Two papers have been published recently concerning the effects of temperature on enzyme secretion from the fungi *Trichoderma reesei*. In the first paper secretion for both a wild-type strain and a hypersecreting strain was found to be higher at 25 °C than at 30 or 35

C when grown on lactose [9]. Similarly, in the second paper two public domain strains showed a four-fold reduction in secretion (normalized to dry cell mass) at 37 °C compared to 17 or 28 °C [10]. The authors of both papers conclude that knowledge of temperature effects on secretion has potential applications in fermentation optimization, but offer no explanations for the observed trends.

Secretion of amylase by the bacteria *Bacillus subtilis* was reported at 30, 40, and 45 °C by Hao, et al [11]. The synthesis of extracellular enzyme was substantially higher at 30 °C than at 40 or 45 °C. An alteration in structure and function of the cytoplasmic membrane was cited by the authors as a possible reason for the lower secretion at higher temperatures

The temperature effects on protein secretion in yeast were reported as early as 1982. Estrela, et al, found that the growth rate and the production of glucoamylase in the yeast *Lipomyces kononenkoae* were independent of growth temperature; while α amylase production had a temperature optimum in the range of 25-28 °C [12].

CONCLUSIONS

It was found that the optimum temperature for growth of SE7-6: pYE αFPHO was 35 °C, while the temperature for optimizing the rate of secretion of S. *pombe* acid phosphatase was determined to be 25 °C. Based on all of the above literature citations, as well as experimentally determined results, it seems to be generally true that over a range of growth temperatures, the optimum temperature for secretion is at a lower temperature that the optimal growth temperature. Whether

this trend is due to a temperature dependence on folding, or due to a thermal dependence on membrane permeability, or due to some other uncharacterized interactions, remains to be seen. What can be concluded is that the best operating temperature for cell growth is not necessarily the best operating temperature for product formation.

ACKNOWLEDGEMENT

The author would like to express his sincere thanks to Professor K. D. Wittrup. Without his ideas, his enthusiasm, his common sense, or most of all his good nature, I wouldn't have been able to even set goals as high as I have already reached.

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APPENDIX A: GROWTH MEDIUM

Liquid SD-CAA All 1X liquid media was diluted from 10X concentrated media for ease of preparation and uniformity. The following table lists component concentrations for 10X and 1X SD-CAA:

Component	10X (g/l)	1X (g/l)
Dextrose (Glucose)	200	20
Sodium Citrate	147	14.7
Yeast Nitrogen Base	67	6.7
Casamino Acids	50	5.0
Citric Acid Monohydrate	42	4.2

When completely dissolved (may require heating), the media was then filter sterilized with a Nalgene disposable filter and stored in the refrigerator. When 1X media was needed, the concentrate was diluted ten fold with Milli-Qed water and filter sterilized.

The casamino acids concentration in the 1X medium, 0.5 %, was determined experimentally to be the lowest concentration to achieve good growth. The 1X media was buffered at pH = 5.0 with a citrate buffer incorporated into the components.

Plates and Slants of SD-CAA The same concentrations of 1X liquid media are used only 2% agar (20 g/l) is added to the recipe.

APPENDIX B: ASSAYS

Growth Rate Measurement The following is a procedure for measuring the maximum specific growth rate.

1. Notes: The Klett meter must warm up for at least 10 minutes before any accurate readings can be taken from it. Also, before inoculating the 50 ml flask with overnight culture, a Klett of the sterile media should be obtained. This reading is to be subtracted from all subsequent readings.

2. Taking Klett Readings: Zero the Klett with water. Wipe any fingerprints off of the side-arm of the flask with a Kimwipe and fill it with culture (or media) by tilting the flask. Put the side-arm into the Klett, adjust knob until arrow lines up with line and record reading. Take several time points (about every half hour) during exponential phase (25-100 Klett). Before exponential growth, there is a lag phase which can vary depending on experimental conditions from approximately 8 to 16 hours for SE7-6: pYE and 12 to 20 hours for SE7-6: pYE αFPHO.

3. Growth Rate: The slope of the linear portion of the graph of ln(Klett) vs Time (hr) is the maximum growth rate, μ_m, in $\frac{1}{hr}$.

Acid Phosphatase Assay The following is a detailed description of the steps involved in obtaining the rate of extracellular AP production:

1. Before any experiments are started, prepare the following solutions:

   25% Trichloro Acetic Acid $\left(\frac{250\text{ g TCA}}{1000\text{ ml }H_2O}\right)$

   pH = 4.0 Acetate Buffer

   (1.65 parts 50 mM CH_3COOH : 1.0 parts 50 mM $CH_3COO^-Na^+$)

   Saturated Sodium Carbonate

   About a half hour before taking a sample, prepare a 2 mg/ml solution of p-NPP in acetate buffer. Since the p-NPP solution is light sensitive, make only what is necessary to complete the experiment. A good approximation is 25 ml per shake flask.

2. At about the same time the p-NPP solution is being prepared, the following equipment should be turned on:

   1. Shimadzu spectrophotometer After the machine is done initializing, it should be set for attachment mode (9). On the next screen choose CPS Data Print (4). Finally, on the next screen, set the processing wavelength (5) to 435 nm.

2. CPS Cell Temperature Controller Set to 20 C by turning off, adjusting setpoint knob, and turning back on.

3. Lauda Constant Temperature Water Bath. Set 0 °C by turning temperature select knob to "&, pressing and holding "&, and adjusting the "&" knob.

3. Make sure plenty of glass pipets, disposable cuvets, and mirero-centrifuge tubes are available. In the warm water bath prewarm eight Eppendorf microcentrifuge tubes filled with 0.6 ml of the p-NPP solution. Prepare an ice bath with ice and water in a gray, 5 x 10 Eppendorf tray. Fill a cuvet with 3-4 ml of the p-NPP, and place it in the spot for the blank in the Shimadzu.

4. Measure and record the Klett reading. Samples should be taken at Klett = 25, 50, 75, and 100. If Klett < 50 then take a 1.0 ml sample; if Klett > 50 then take a 0.5 ml sample. Centrifuge the sample for one minute at 14000xG in the Eppendorf micro-centrifuge in the glass door refrigerator. With a pipet, carefully draw off the supernatant and save it in another tube. Resuspend the cells in acetate buffer according to the following equation:

$$\text{Resuspend Vol.} = \frac{(\text{sample vol.})*(\text{Klett reading})}{50}$$

to obtain the equivalent Klett of 50. Now we have two Eppendorf tubes: one with the supernatant and one with the resuspended cells.

5. Set two timers with ten and twenty minutes on both. Take four of the prewarmed p-NPP solutions out of the temperature bath. Using the Eppendorf repeater pipettor with the 0.5 ml Combitip and a dial setting of 5 to dispense 0.05 ml, quickly dispense 0.05 ml of the supernatant into two of the prewarmed tubes and press 10 minutes on the timer. Repeat for the other two prewarmed tubes except press 20 minutes. Close the lids on all of the tubes, vortex, and place in the constant temperature bath. Repeat this sequence with the remaining four centrifuge tubes containing the p-NPP solution, except use the resuspended cells instead of the supernatant. Make sure the cells are suspended well, i.e. vortexed, before pipetting them.

6. When the first timer goes off, take out the two 10 minute supernatant samples, place in the ice bath, and add 0.7 ml saturated Na_2CO_3 and 0.15 ml of 25% TCA. Vortex these samples and place in the ice bath. About one minute later, the two 10 minute cell-associated samples will be ready. Repeat the above sequence (ice bath, etc.) for the two cell-associated samples. Find four clear (no scratches) cuvets and transfer the contents of each of the tubes in the ice bath to a cuvet, remembering which samples are which.

Add 1.4 ml of saturated Na_2CO_3 to each cuvet, mix by pipetting in and out several times, wipe with a Kimwipe, and load in the Shimadzu. Cells 1 through 6 are labelled from the front to the back of the machine. Press start and the data will be printed out. Label the printout. In about 5 minutes the 20 minute timers will go off. Repeat the above procedure exactly for the 20 minutes samples.

Plasmid Contain Fraction At Klett = 50, the following test should be performed:

Pipet 0.01 ml of culture into 10 ml of Milli-Qued water. This is a 1000X dilution. Next, pipet 1.0 ml of this diluted cell suspension into 9.0 ml of Milli-Qued water. The original cell culture has now been diluted 10,000X. Smear 0.2 ml of this dilution onto a high-phosphate YPD plate. Place in the incubator and grow for approximately 1-2 days until colonies are big enough to count. Count the colonies (there should be about 500 colonies on the plate). Add about 0.25 g of agar to 15 ml of acetate buffer (per plate), autoclave, and cool to 50 °C. Dissolve in this agar solution 30 mg each of α-naphthyl phosphate and fast garnet. Pour over the colonies on the high phosphate plates. Count the colonies that turn dark red. The plasmid containing fraction is the number of red colonies divided by the total number of colonies.

APPENDIX C: FIGURES

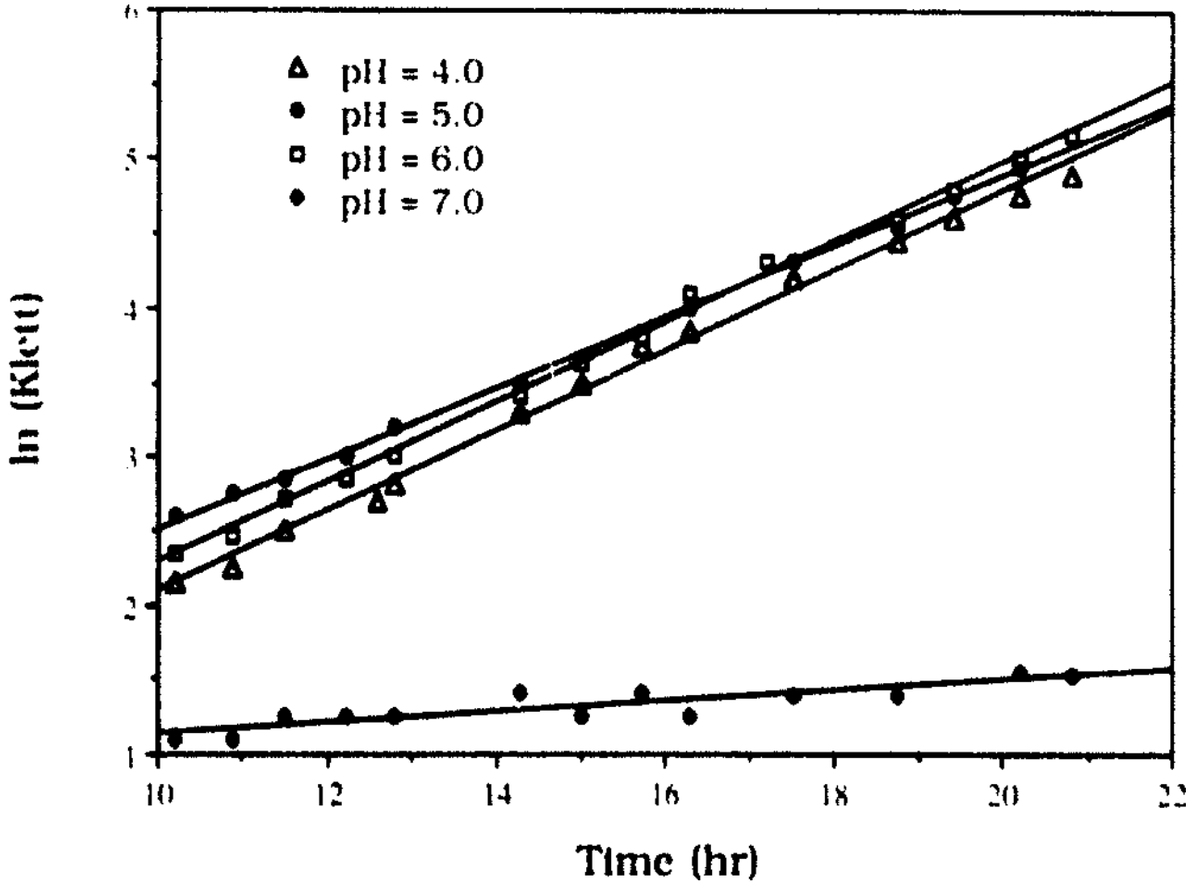

Figure 1. The Effect of pH on the Growth of SE7-6: pYE αFPHO

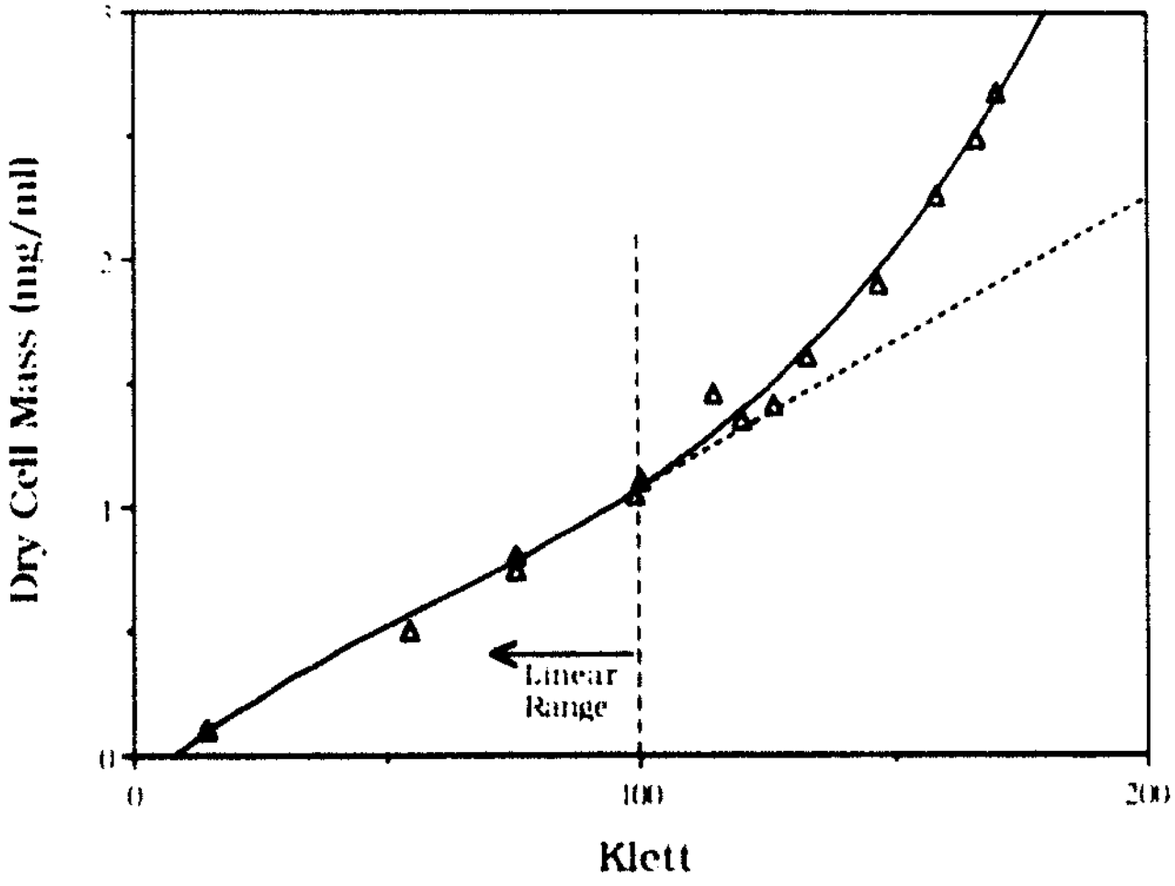

Figure 2. Correlation Between DCM and Klett Reading

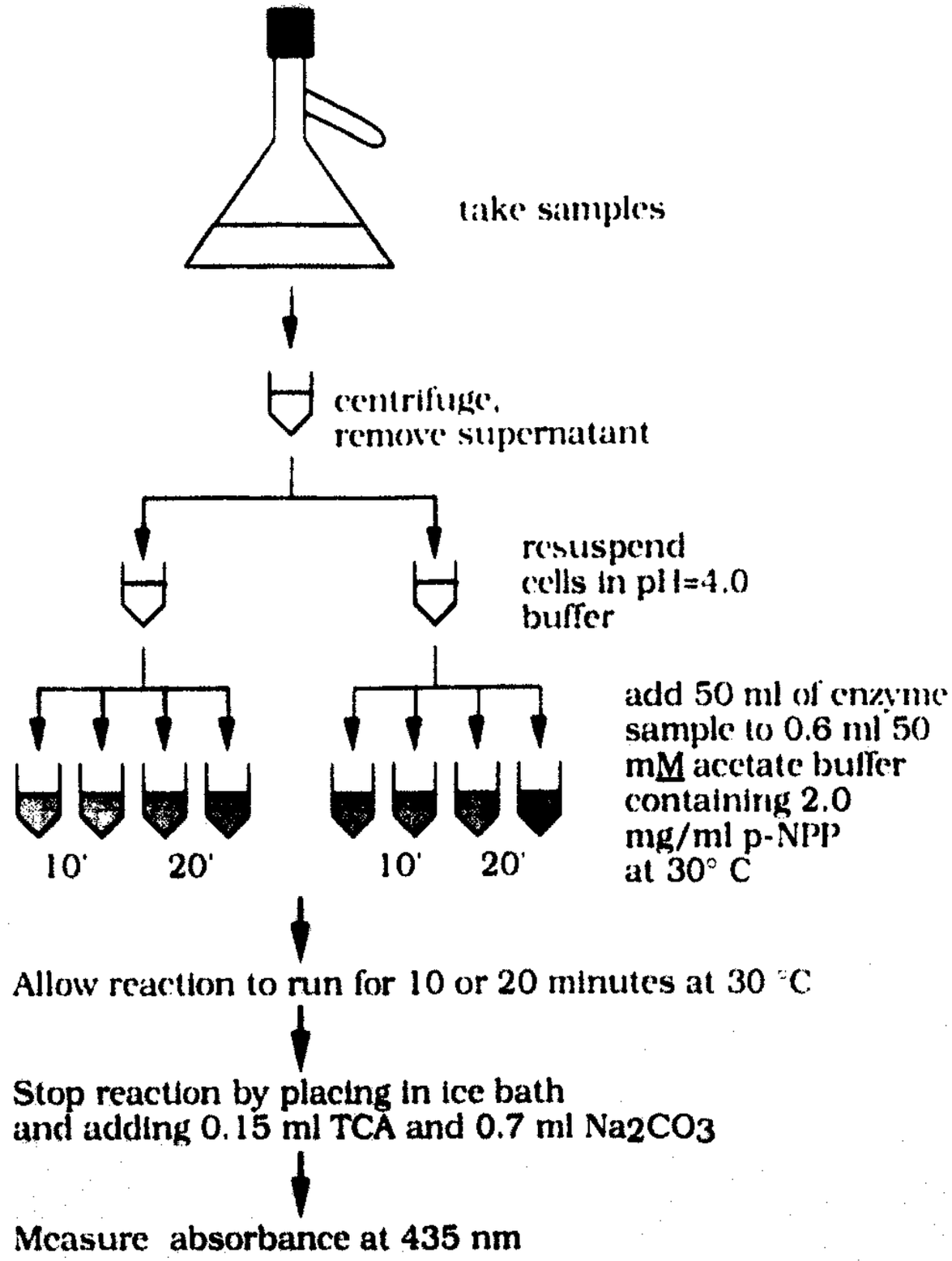

Figure 3. Protocol for Acid Phosphatase Assay

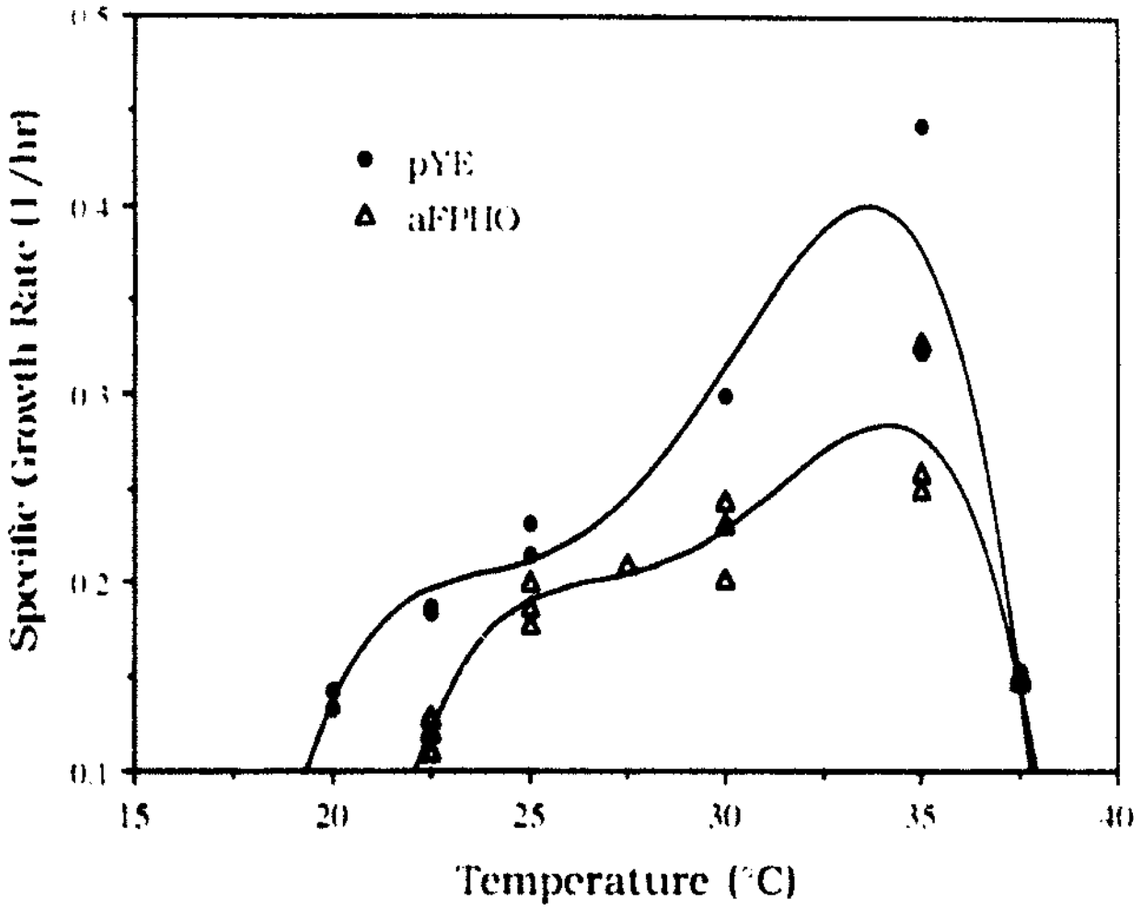

Figure 4. A Comparison of Growth Rates
Between pYE and αFPHO

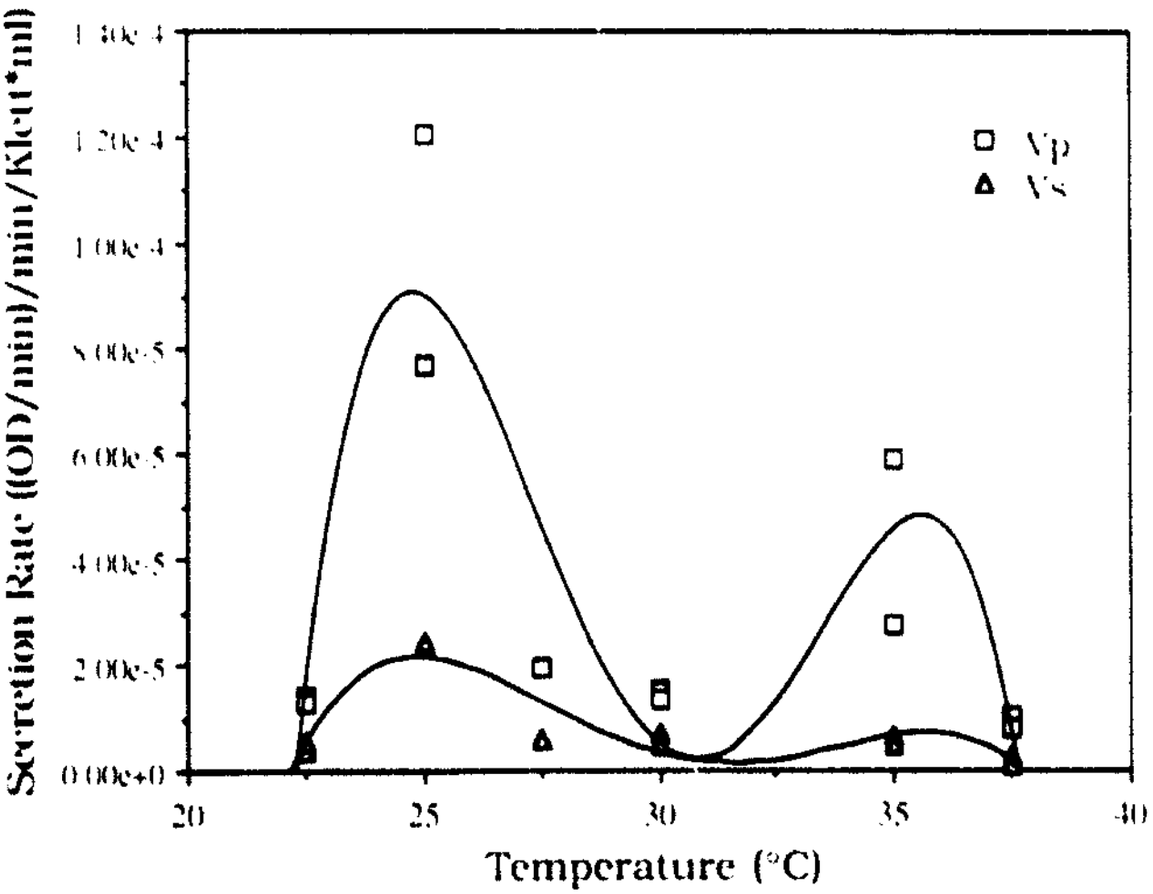

Figure 5. A Comparison of the Effects of Temperature on Total (Vp) and Extracellular (Vs) Secretion Rates