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# A Kinetic Study of the Fermentation of Cane Sugar Using *Saccharomyces cerevisiae*

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## ABSTRACT

**The fermentation of cane sugar as substrate by *Saccharomyces cerevisiae* (enzyme) was critically investigated to obtain certain useful kinetic parameters and to determine the effect of temperature, pH, substrate and yeast (enzyme) concentration on the rate of fermentation. The results indicate that the rate of fermentation (measured as rate of production of $CO_2$) increased in proportion with temperature (optimum 32˚C - 36˚C), pH (optimum 5.5) substrate (optimum 50 v/v%) and yeast concentration (optimum 3.5 - 4.5 w/v%) up to a limit and subsisted either as a plateau and/or, decreases as the case may be. This suggests that the reaction takes place in two steps. The kinetic parameters examined are maximum rate of reaction $V_{max}$ ($2.0 \times 10^2$ M·min$^{-1}$), catalytic constant, $k_2$ ($1.81 \times 10^{-1}$ min$^{-1}$), overall rate constant, k ($1.53 \times 10^1$ min$^{-1}$), order of initial reaction (approx. first order), dissociation constant of enzyme-substrate complex, $k_s$ ($2.74 \times 10^3$), Michaelis constant, $k_m$ ($2.74 \times 10^3$ M), and the specific activity of enzyme on substrate concentration ($1 \times 10^{-1}$ w/v%). The result of this study showed that the equilibrium step involving $k_{-1}/k_1$ is the limiting step deciding the direction of reaction as well as the specific activity of the enzyme.**

## KEYWORDS

**Fermentation; Substrate Concentration; Kinetics; Cane Sugar; Enzyme**

## 1. Introduction

The fermentation of biomass using suitable enzymes to obtain alcohol had in recent years attracted the attention of stake holders in the industrial sector. Alcohols from vegetable sources are useful as renewable components of bio-diesel as well as for the production of a range of useful chemicals such as surfactants, solvents for paints, vanishes, inks and adhesives [1,2]. In addition, alcohols are used for medicinal, pharmaceutical, flavoring and beverage purposes. Ogunye and Susu [3] had conducted fermentation studies on pineapple juice, while Holberg [4] investigated on grape juice. The kinetic study of the fermentation of cane sugar molasses had also been done by Digwo *et al*. [5]. In all of these, the required kinetic information for industrial production is lacking. However, the published nutritive value of cane sugar juice per 28.35 g of sugar cane juice includes: energy (111.13 kJ), carbohydrates (27.5 g), protein (0.27 g), calcium (11.23 mg), iron (0.37 mg), potassium (41.96 mg), and sodium (17.01 mg) [6]. A major feature of enzymes is that they are extremely effective as catalysts-providing alternative and simple routes much more than chemical catalysts [7]. The catalytic action arises from the substantial reduction of the activation energy. Moreover, the catalytic activity is localized in certain amino acids residues in the protein enzyme which provide suitable sites for reaction in a lock and key system with substrate. Enzyme catalyzed reaction is usually characterized by a very high rate value of the order of $10^4$ to $10^9$ and high specificity. The determination of the specific activity of *Saccharomyces cerevisiae* with respect to cane sugar is the main objective of this study.

### Theory of Fermentation Kinetics

The theory of fermentation kinetics is well established

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and documented in literature. It is adapted in this report to suite this investigation. In an enzyme [E] catalyzed reaction involving a single substrate [S], the rate varies linearly with the substrate concentration at low concentration (first order kinetics) and at high concentration; the rate becomes independent of substrate concentration (zeroth order kinetics). On this basis, the reaction scheme can be stated in terms of the mechanism.

$$S + E \underset{K_2}{\overset{K_1}{\rightleftarrows}} ES \xrightarrow{K_2} P + E \quad (1)$$

where [E] = enzyme (yeast), [S] = substrate, [ES] = enzyme-substrate complex, and [P], the product. Further $K_1$ is the rate constant for the formation of [ES] complex, $K_{-1}$ the decomposition of ES complex to E and S, and $K_2$, the catalytic constant or the turnover number of an enzyme. The first step is a rapid equilibrium and the second step a slow, rate determining step. Since in most enzyme reaction, the substrate is much greater than that of the enzyme and the concentration of [ES] is much less than that of the substrate [8], applying the steady state approximation for the [ES] complex is given by:

$$\mathrm{d}[ES]/\mathrm{d}t = 0 = k_1[E][S] - k_1[ES] - k_2[ES] \quad (2)$$

While the rate of formation of product ($R_p$) is:

$$v_o = R_p = k_2[E]_o[S]/k_m + S \quad (3)$$

where $K_m$, the Michaelis constant is given by

$$k_m = k_{-1} + k_2/k_1 \quad (4)$$

The maximum or limiting rate ,$V_{max}$ of the reaction is considered to be the rate at which all the enzyme molecules are in the complex form [ES] *i.e.* $[E]_o$ = [ES]. It follows that $V_{max}$ is proportional to $[E]_o$.

$$V_{max} = k_2[E]_o \quad (5)$$

Substituting Equation (5) in Equation (3) gives

$$R_p = v_o = V_{max}[S]/k_m + [S] \quad (6)$$

From Lineweaver and Burk [9], the reciprocal of Equation (6) is:

$$1/v_o = k_m/V_{max}[S] + 1/V_{max} \quad (7)$$

where $V_{max}$ and $k_m$ can be evaluated from a plot of $1/v_o$ versus 1/[S] while $k_2$ is in principle obtained from the plot of $V_{max}$ versus $[E]_o$ (Equation (5)). Moreover, Michaelis and Menten [10] also considered that the initial rate of product formation $v_o$ is given by;

$$v_o = \mathrm{d}[p]/\mathrm{d}t = k_2[ES] \quad (8)$$

On the basis of the rate determining step and equilibrium values of [E] = $[E]_o$ – [ES]; $[S]_o$ – [ES] at time t. From a consideration of equation 4, if $k_{-1} >> k_2$, therefore $k_m = k_s = k_{-1}/k_1$ can be treated as a dissociation constant ($k_s$) *i.e.*: $k_s = k_{-1}/k_1$ = [E][S]/[ES]

$$k_s = k_{-1}/k_1 = ([E]_o - [ES])[S]/[ES] \quad (9)$$

Expanding and dividing through by [ES] gives:

$$[ES] = [E]_o[S]/k_s + [S] \quad (10)$$

Substitution of Equation (10) into Equation (8) gives:

$$v_o = \mathrm{d}[p]/\mathrm{d}t = k_2[E]_o[S]/k_s + [S] \quad (11)$$

The reciprocal of $v_o$ gives: $1/v_o = k_s + [S]/k_2[E]_o[S]$

$$1/v_o = k_s/k_2[E]_o[S] + 1/k_2[E]_o \quad (12)$$

A plot of $1/v_o$ versus 1/[S] yields $k_2$ and $k_s$ from a consideration of the slope and intercept. A consideration of Equation (7) and (12) proves that: $V_{max} = k_2[E]_o$.

It must be noted that, $k_m$ can be equated with $k_s$ only when $k_{-1} >> k_2$. In this condition, $k_m$ is considered as a measure of the strength of the [ES] complex:

$$[ES] \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [E] + [S]$$

and $k_m = k_{-1}/k_1$ from Equation (4).

When $k_1$ is very small, $k_m$ is large indicating a weak binding between enzyme and substrate [10].

## 2. Materials and Methods

### 2.1. Experimental Materials

Sugarcane was purchased from Iruekpen market, Ekpoma, Edo State, South-South, Nigeria. pH meter standardized with appropriate buffer solutions (buffer 4) and yeast (*Saccharomyces cerevisiae*) manufactured by Vahine professional, Mc cormick, France SAS was used as received. Fermentation vessels, vials and thermostated water baths were also used for the experimentation.

### 2.2. Preparation of Sugar Cane Juice

The sugarcane was washed, peeled, chopped into bits or pieces, and pounded in a mortar. The fibers were then manually removed by squeezing out the juice from it by hand. The juice obtained was sterilized by heating in an aluminum can at temperature of 90˚C for 40minutes and then cooled. After cooling, 3000 $cm^3$ was obtained from 5.632 kg of cane. The juice was filtered and treated with a 3% Sodium metabisulphite, ($Na_2S_2O_5$) to inhibit the growth of any undesirable type of microorganism such as acetic acid bacteria, wild yeast and mould [11]. Thereafter the required quantity of juice was transferred into the fermentation vessels.

### 2.3. Experimental Procedure

The fermentation vessels were washed and later sterilized with a 3% solution of sodium metabisulphite for 5 minutes. 1000 $cm^3$ of the juice was properly conditioned by sterilizing at 90˚C and brought to the required pH with

either 0.1 M HCl or 0.1 M NaOH. Seven vials containing substrate were prepared for each of seven sampling times at 0, 30, 60, 90, 120, 150, 180, and 210 (min). The samples were fermented in the sealed polymeric vial with connected tubes for the estimation of gas production. Ig of yeast was added to each of the fermenter. The substrate and yeast were properly mixed by shaking and the yeast allowed activating for 20minutes. The escape of $CO_2$ was prevented by sealing the air inlet with a cresol-perfumed jelly. The $CO_2$ produced in each sealed vial was collected in water and measured by titration, with 0.IM solution of NaOH using phenolphthalein indicator.

### 2.3.1. Determination of Effect of Temperature

The effect of temperature on fermentation kinetics was determined by keeping other factors such as substrate concentration, pH of the juice, yeast concentration, and fermentation time constant.

The temperature was varied between 30˚C - 42˚C, using a thermostated water bath.

### 2.3.2. Determinaton of Effect of Substrate Concentration

In determining the effect of substrate concentration on fermentation kinetics, all other factors such as temperature, pH, yeast concentration, and fermentation time were kept constant. The substrate concentration was varied between 20 - 80 (v/v%).

### 2.3.3. Determination of Effect of PH

Substrate concentration, temperature, fermentation time and yeast concentration were kept constant in determining the effect of pH on fermentation kinetics. The pH meter was standardized with a buffer 4 solution. The pH was varied by the introduction of 0.1 M $H_2SO_4$ or 0.1 M NaOH solution to the required pH value and measured by a pH meter. The pH of the juice was varied between 3.0 - 6.0 (pH).

### 2.3.4. Determination of Effect of Yeast Concentration

The effect of yeast concentration on fermentation was determined by varying yeast concentration between 1 - 7 (w/v%).

### 2.3.5. Determination of Rate of Fermentation

The rate of fermentation was measured as the volume of $CO_2$ produced at 30 minutes' interval of time.

## 3. Results and Discussion

The data on the effect of temperature, substrate concentration, pH, and yeast concentration on the fermentation of cane sugar with *Saccharomyces cerevisiae* are presented in **Tables 1-4**.

**Table 1. Effect of temperature on fermentation rate using 50 (v/v%) subtrate, yeast 1.0 (w/v%), and pH 5.0.**

| Time (min) | Volume of $CO_2$ Produced ($cm^3$) Temperature (˚C) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 30 | 32 | 34 | 36 | 38 | 40 | 42 |
| 30 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 60 | 142 | 128 | 128 | 128 | 171 | 128 | 142 |
| 90 | 200 | 142 | 142 | 142 | 200 | 200 | 157 |
| 120 | 242 | 157 | 171 | 157 | 242 | 214 | 171 |
| 150 | 271 | 171 | 242 | 200 | 300 | 228 | 200 |
| 180 | 300 | 200 | 300 | 228 | 457 | 242 | 242 |
| 210 | 328 | 228 | 342 | 300 | 500 | 257 | 271 |
| Rate($M \cdot min^{-1}$) | 0.70 | 1.30 | 1.80 | 2.00 | 1.50 | 0.90 | 0.90 |

**Table 2. Effect of substrate concentration on fermentation rate using 1.0 (w/v%) yeast, at 30˚C and pH 5.0.**

| **Volume of $CO_2$ produced ($cm^3$)** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Time (min) Substrate concentration (v/v%)** | | | | | | | |
| | 20 | 30 | 40 | 50 | 60 | 70 | 80 |
| 30 | 185 | 242 | 114 | 185 | 200 | 185 | 114 |
| 60 | 271 | 257 | 214 | 242 | 242 | 257 | 214 |
| 90 | 328 | 285 | 342 | 400 | 271 | 285 | 257 |
| 120 | 528 | 314 | 371 | 800 | 300 | 800 | 371 |
| 150 | 571 | 828 | 400 | 842 | 342 | 828 | 414 |
| 180 | 600 | 857 | 700 | 942 | 371 | 842 | 714 |
| 210 | 800 | 971 | 785 | 971 | 400 | 942 | 728 |
| Rate($M \cdot min^{-1}$) | 1.40 | 3.40 | 4.50 | 4.90 | 4.10 | 3.70 | 3.40 |

**Table 3. Effect of pH on fermentation rate using 50 (v/v%) subtrate, 1.0 (w/v %) yeast, at 30°C.**

| **Volume of $CO_2$ produced ($cm^3$)** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **pH** | | | | | | |
| **Time (min))** | 3.0 | 3.5 | 4.0 | 4.5 | 5.0 | 5.5 | 6.0 |
| 30 | 442 | 271 | 228 | 200 | 200 | 271 | 200 |
| 60 | 471 | 400 | 400 | 257 | 214 | 328 | 300 |
| 90 | 600 | 442 | 500 | 300 | 400 | 385 | 342 |
| 120 | 642 | 528 | 557 | 328 | 428 | 442 | 357 |
| 150 | 828 | 557 | 571 | 342 | 528 | 500 | 400 |
| 180 | 928 | 714 | 600 | 428 | 585 | 542 | 500 |
| 210 | 985 | 928 | 657 | 500 | 671 | 571 | 628 |
| Rate ($M \cdot min^{-1}$) | 1.90 | 2.30 | 2.40 | 2.60 | 3.00 | 4.00 | 2.50 |

**Effect of temperature:** The data on the effect of temperature on the rate of fermentation is shown in **Table 1** and plotted on **Figure 1** as rate of fermentation versus temperature (˚C). It is observed that the rate of production of $CO_2$ increased up to 36˚C and then later decreased. The results further showed that though there is a wide range of temperature over which the enzyme is active, there is a narrow range of temperature (35˚C - 36˚C) over which the enzyme activity is a maximum. The shape of

**Table 4. Effect of yeast concentration on rate of fermentation using 50 (v/v%) substrate, at 30˚C, and pH 5.0.**

| Volume of $CO_2$ produced ($cm^3$) | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Yeast concentration (w/v%) | | | | | | |
| **Time (min)** | **1** | 2 | 3 | 4 | 5 | 6 | 7 |
| 30 | 285 | 100 | 100 | 100 | 100 | 100 | 100 |
| 60 | 428 | 128 | 128 | 128 | 114 | 114 | 114 |
| 90 | 600 | 142 | 142 | 142 | 128 | 142 | 142 |
| 120 | 628 | 157 | 157 | 157 | 142 | 171 | 200 |
| 150 | 771 | 200 | 171 | 271 | 200 | 300 | 300 |
| 180 | 800 | 228 | 200 | 400 | 300 | 457 | 400 |
| 210 | 885 | 300 | 300 | 428 | 328 | 700 | 500 |
| Rate ($M{\cdot}min^{-1}$) | 1.20 | 1.30 | 1.50 | 2.00 | 1.80 | 1.10 | 1.10 |

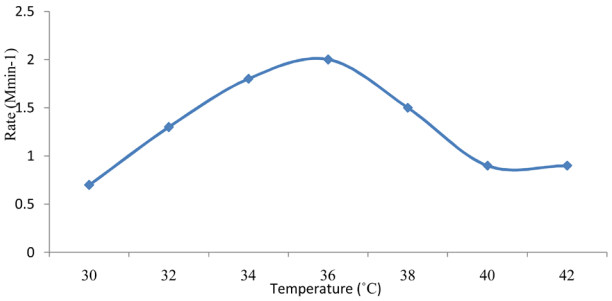


**Figure 1. Variation of rate of fermentation with temperature of substrate using 50 (v/v%) substrate, yeast 1.0 (w/v%), and pH 5.0.**

the plot is generally hyperbolic. The plot signaled a two step reaction which is corroborated by the rapid release of $CO_2$ at the initial stage of the fermentation, followed by a decrease or slow release of the gas. The initial increase in rate with temperature is expectedly a function of the increase in the average kinetic energy of the molecules. However, a further increase in temperature beyond 36˚C would trigger the breakdown of the enzymatic structure due to increased thermal vibration of the enzyme.

**Effect of substrate concentration:** The data on the effect of substrate concentration on rate of fermentation is shown in **Table 2** and plotted on **Figure 2**. The figure showed that the rate of fermentation varied in proportion with substrate concentration up to 50 (v/v%). However, further increase in the substrate concentration showed no effect on the rate of reaction. This suggests that at the initial stage of the reaction, all active sites of the enzyme were saturated and therefore further increase in substrate concentration could not lead to further increase in the rate of fermentation. The plot indicates that there is a wide range of concentrations over which the enzyme is active.

**Effect of pH:** The rate of fermentation increased in relation with pH (*i.e.*, from 3.0 - 5.5). The data is shown in **Table 3** and plotted as rate of fermentation versus pH

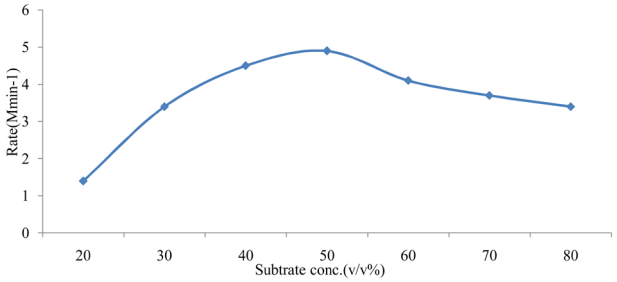


**Figure 2. Variation of rate of fermentation with substrate concentration using 1.0 (w/v%). Yeast, at 30˚C and pH 5.0.**

(**Figure 3**). This result is in conformity with the optimal pH range of *Saccharoymces cerevisiae* 4.5 - 5.5 [12]. Outside the optimum pH range, the enzyme cells are less tolerant to the pH environment and expectedly less active and less efficient in substrate conversion. The figure indicates that there is a narrow range of pH over which the enzyme activity is a maximum.

**Effect of yeast concentration:** The data on the effect of yeast concentration on the rate of fermentation is shown in **Table 4** and plotted in **Figure 4** as rate of fermentation versus yeast concentration (w/v%). It is seen that though there is a wide range of concentration over which the yeast (enzyme) is active, there is a narrow range over which the activity is a maximum. It is considered that at high yeast concentration, the substrate becomes unavailable for the large population of yeast for a particular enzyme-substrate system. This suggests that there is a fixed or particular amount of substrate that can complex with the yeast. That complexation is controlled by the system thermodynamics. Further increase in yeast concentration therefore had no effect on the rate.

In **Tables 5** and **6** are shown the kinetic parameters: overall rate constant k, order of initial reaction n, maximum rate of fermentation $V_{max}$, catalytic constant, $k_2$ also known as the turnover number, dissociation constant for the enzyme-substrate complex $k_s$ the Michaelis constant $k_m$, and the specific activity of the enzyme. It is seen (**Table 5**) that the overall rate constants k with respect to temperature is (1.35 $min^{-1}$); substrate concentration (1.53 $min^{-1}$); yeast concentration (1.13 $min^{-1}$) and pH (1.87 $min^{-1}$); while the order of initial reaction is approximately first order. **Table 5** further indicates that the rate of the reaction is highly dependent on the substrate concentration and that all other conditions such as temperature, yeast concentration and pH have seemingly low dependence of the rate, but rather a limiting one at optimum range.

Most importantly, the kinetic parameters highly valued in fermentation process are shown in **Table 6**. The maximum rate of fermentation $V_{max}$ being $2.0 \times 10^2$ $M{\cdot}min^{-1}$. The value represents the maximum velocity attainable. The catalytic constant $k_2$ is $1.81 \times 10^{-1}$ $min^{-1}$,

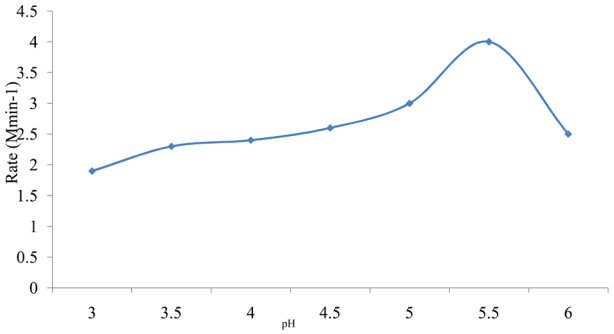


**Figure 3. Variation of rate of fermentation with ph of substrate using 50 (v/v%) substrate, 1.0 (w/v%), at 30˚C.**

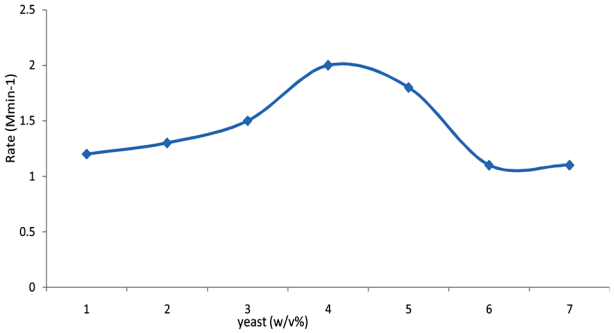


**Figure 4. Variation of rate of fermentation with yeast concentration (w/v%) using 50 (v/v%) substrate, at 30˚C and pH 5.0.**

**Table 5. Values of k and n for the fermentation of cane sugar using *Saccharoymces cerevisiae.***

| | RATE CONSTANT k | ORDER OF REACTION, n |
|---|---|---|
| TEMPERATURE | 1.35 | Nil |
| SUBSTRATE CONCENTRATION | 1.53 | 0.66 (approx.1$^{st}$ order) |
| YEAST CONCENTRATION | 1.13 | Nil |
| pH | 1.87 | Nil |

**Table 6. Kinetic parameters for the fementation of cane sugar using *Saccharomyces cerevisiae.***

| PARAMETER | VALUE |
|---|---|
| MAXIMUM RATE, $V_{MAX}$ ($M \cdot min^{-1}$) | $2.0 \times 10^{2}$ |
| CATALYTIC CONSTANT $K_2$ ($min^{-1}$) | $1.81 \times 10^{-1}$ |
| DISSOCIATION CONSTANT $K_S$ | $2.74 \times 10^{3}$ |
| MICHAELIS CONSTANT $K_m$ (M) | $2.74 \times 10^{3}$ |
| SPECIFIC ACTIVITY (*S. cerevisiae*) | $1 \times 10^{-1}$ |

which is the number of substrate molecules converted into products per unit time. The dissociation constant $k_s$ is $2.74 \times 10^3$ of the enzyme-substrate complex. The Michaelis constant $k_m$ is $2.74 \times 10^3$ M, which is usually deployed to characterize a particular enzyme-substrate system. $k_m$ is equal to the concentration of substrate required to give half the maximum velocity. The value of $k_m$ in this investigation is quite large (**Table 6**) with reference to known $k_m$ values that lie between $10^{-1}$ and $10^{-6}$ M[13]. The large value indicates that the binding between enzyme and substrate is very weak with respect to cane sugar and *Saccharoymces cerevisiae* [E]. The large value of $k_m$ obtained can only be possible if the decomposition constant $k_{-1}$ of ES to E + S is much greater than $k_2$, the catalytic constant as seen from the data in **Table 6**; and as well if the formation rate constant $k_1$ of ES complex from E and S is very small. That $k_{-1} >> k_2$, therefore $k_m = k_s = k_{-1}/k_1$ confirms the values of $k_m$ ($2.74 \times 10^3$) and $k_s = 2.74 \times 10^3$ shown in **Table 6**. Given the value of $k_2$ ($1.81 \times 10^{-1}$ $min^{-1}$) in **Table 6**, $k_2$ often lies between 0.5 and $10^4 S^{-1}$ [13]. It indicates that the equilibrium step involving $k_{-1}$ is more thermodynamically favored than step 2 that involves $k_2$ (the catalytic constant). The specific activity is the units of enzyme activity per gram of protein (yeast). It is found to be $1 \times 10^{-1}$. A unit of enzyme is taken to be the amount which will catalyze the reaction of a unit of substrate per minute. Thus specific activity in relation to enzyme is the ratio of enzyme activity to the total weight of enzyme present in the mixture. This was obtained by cross plotting the rates of fermentation with respect to enzyme and yeast concentrations.

## 4. Conclusion

The cross plots had shown that at maximum rate, 13 w/v% yeast is required to ferment 44 v/v% substrate; while at half maximum rate, 5 w/v% yeast ferments 27 v/v% of substrate. This information is practically important for fermentation process engineers.

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