Chapter V

DISCUSSION

5.1 Part I Cultivation of *Candida utilis* in Shake Flask Apparatus

The analyses of pineapple juice by Lane-Ennon method showed that the sugar content of the pineapple juice obtained in this study expressed as percent invert sugar varied from 11-14%. The composition of pineapple juice depends upon variety, ripeness, season, crop, locality, plant status, weather, fruit portions used and of blemishes (Tressler and Joslyn, 1971). They has also reported that the main sugars of pineapple juice are sucrose and other reducing sugars i.e., fructose and glucose. So pineapple juice can serve well as energy source for yeast growth. It appeared that *C. utilis* growed poorly on the pineapple juice alone while the addition of 0.5% (w/v) ammonium sulfate to pineapple juice in the medium 8 increased the growth (Figure 2). This indicated that the juice is lacking nitrogen source. The results agree with the work of Stevenson and Shannon (1975) who reported that the addition of nitrogen to substrate increased the growth of yeast. The needed nitrogen is utilized principally as building material for synthesis of protein (Nickerson and Rose, 1956; Nolte et al., 1942; Morris, 1958). Ammonium sulfate does not provide only the source of nitrogen

but the yeast can take up the sulfur from the sulfate too. Most yeasts take up the sulfur they need from inorganic sulfate (Suomalainen and Oura, 1971). Sulfur is required for the synthesis of sulfur-containing amino acids, i.e., cystine, cysteine, and methionine; and of sulfur-containing essential organic cell components, i.e., thiamin, biotin, etc. (Nickerson and Rose, 1956; Rose, 1961; Morrison, 1958). Hence, the supplement of ammonium sulfate to pineapple juice provides both the nitrogen and sulfur sources. Ammonia and ammonium salts particularly ammonium sulfate and diammonium phosphate have been found to be the most suitable sources of nitrogen on account of their availability, low cost, and ready assimilation (Prescott and Dunn, 1959; Morris, 1958). The improvement of the yeast growth on the addition of phosphate into the medium containing the pineapple juice and ammonium sulfate was obtained (Figure 3). Phosphates have been found to serve several purposes for yeast growth. They supply phosphorus necessary for synthesis of nucleoproteins which the yeast elaborate during the growth. They are also assisted in buffering the wort during propagation. This is very important to produce yeast of good quality and to obtain maximum yield of yeast from raw materials used (Nolte et al., 1942; Nickerson and Rose, 1956; Morris, 1958; Suomalainen and Oura, 1971). Potassium was found to be necessary upon yeast growth. (Prescott and Dunn, 1959; Suomalainen and Oura, 1971). In the presence of potassium, the sulfur was taken up faster by

the cell than in the absence of potassium. The fixation of sulfate in the cell was found to promote by potassium (Werk, 1959). The presence of potassium caused an earlier and more intensive formation of methionine. The formation of protein was at first promoted by potassium. Potassium uptake paralleled polysaccharide synthesis (Werk, 1959; Suomalainen and Oura, 1971). Phosphorus and potassium are the principal mineral requirements of the yeast organism (Frey et al., 1936 Nolte et al., 1942; Reiser, 1954). Molasses and grains, the main raw materials for the manufacture of yeast, were found to contain sufficient potassium but may be deficient in phosphorus. It was thus sometimes necessary to furnish additional phosphorus, and this was usually added in the forms of ammonium or calcuim-phosphate (Frey et al., 1936; Nickerson and Rose, 1956; White, 1954). Phosphorus, in the form of P_2O_5, was added in molasses or wood hydrolyzates (Peppler, 1970, Reed and Peppler, 1973). Phosphoric acid was added as supplemental nutrient in potato starch wastes (Oosten, 1976), and super phosphate in vegetable hydrolyzates (Saviny et al., 1967). Potassium requirement was also supplemented in the form of KCl (Savinykh et al., 1967; Peterson et al., 1945; Synder, 1970). In the production of C. utilis from rice husks, $(NH_4)_2SO_4$, KCl, and super phosphate were required for yeast nutrition (Savinykh et al., 1967). In this study, potassium dihydrogan phosphate was used because this salt was found to absorb readily in the form of monovalent ion,

$H_2PO_4^-$ (Suomalainen and Oura, 1971). Besides, this soluble phosphate can provide the potassium element for the growth of yeast. In using chemically defined media containing only pure chemical compounds, it has been shown that microorganisms .. require the followings :(a) a utilizable source of energy and carbon, (b) a utilizable source of nitrogen, and (c) certain inorganic salts. In addition, some microorganisms, including most yeasts require various organic compounds (intact form) such as growth factors, which the cell is not able to synthesize (Nickerson and Rose, 1956). The growth could be induced when extracts of yeasts or the supernatant fluid from old yeast cultures were added to the basically simple medium (Nickerson and Rose, 1956; Morris, 1958). Yeast extract is, the water soluble portion of autolyzed yeast, an exellent sources of B-complex vitamins (Morris, 1958, Difco Manual, 1953; Baltes, 1971). The vitamins required by a large numbers of yeast are biotin, thiamin, pantothenic acid, inositol, nicotinic and pyridoxine (Nickerson and Rose, 1956; Morris, 1958; Suomalainen and Oura, 1971; Johnson and Olson, 1949). All of the vitamins mentioned above are not required by yeast altogether. Particular strain of yeast has a specific requirement for each vitamin, for example, Saccharomyces cereviseae has a specific requirement to biotin (White, 1954; Burrows, 1970). However, there has no data available on the specific requirement for C. utilis. It was reported by Kung-Chin and his coworkers (1968) that the addition

of thiamin and calcium panthothenate raised the yield of the yeast as well as dry protein in the media, but the addition of pyridoxine reduced the protein content. It was also found that the presence of each amino acid in the yeast was changed by the addition of vitamins (Kung-Chin et al., 1968). So various studies were carried out in order to establish the effect of yeast extract. In this study, no improvement over the yield of yeast was obtained from the medium 3 compared to that from the medium 1 (see Figure 4 and Table 3). Both of these media contained the same source of sugar, nitrogen and phosphate except that yeast extract was added in the medium 3. The results may be explained in two ways. First, sufficient growth factors may be present already in the medium because the substrate is pineapple juice which is a complex medium containing many known and unknown nutritional growth factors especially thiamin (see Table 1). Thiamin is always required for a large number of yeasts (Fernande, 1968). Second, the presence of yeast extract may not be necessary because many microorganisms are capable to synthesize all the growth factors for their own metabolic needs, with the result that they are independent of the exogenous supply (Rose, 1961). Candida utilis produced, in general, a sufficient amount of growth factors in a synthetic media (Vernerora, 1967), and other medium such as hydrocarbon (Borukaero, 1967). Candida utilis has the ability to synthesize vitamin B complex, nicotinic acid, and glutathione from sugar and mineral

salts in the nutritive medium (Nolte et al., 1942, Oliverira, 1974). Vitamins or yeast extracts did not have any affect on the growth of C. utilis (Rose, 1961). The result obtained in this study agrees well with the works of Synder (1970), and Bunker (1963) who reported that in cultivating C. utilis no additional growth-promoting factors was necessary. The results from Figures 2-4 indicated that the addition of $(NH_4)_2SO_4$ and KH_2PO_4 were required for the growth of C. utilis on pineapple juice. Most of other wastes also required the addition of nutrient nitrogen, phosphorus and potassium for yeast to utilize sugars in the wastes (Reiser, 1954). Harris and coworkers (1948c) found that Torula yeast needed about 3.2 pounds of nitrogen, 1.5 pounds of phosphorus pentoxide, and 1 pound of potassium chloride per 100 pounds of reducing sugar. Higher nitrogen concentrations gave a yeast of higher protein content. Attempt was made to find other nutrient nitrogens to provide better growth than ammonium-sulfate. Yeast was unable to assimilate elementary nitrogen but assimilated ammonia, urea, amino acids and peptone (Frey et al., 1936; Roper, 1970). In this study, the difference in the sources of nitrogen on the growth of C. utilis on pineapple juice was determined (see Figure 5) also with the yeast yield (see Table 3). Apparently, ammonium-sulfate was the preferred nitrogen sources for C. utilis when grown on the pineapple juice. Maximum yield was obtained from the medium 1. The ability of C. utilis to utilize ammonium

(NH_4^+) nitrogen in addition to organic nitrogen was recognized. The result can be explained on the basis of the properties of these nutrient materials. It was found by Uden et al., (1966) who studied the utilization of nitrogen compound by Torulopsis. Fourteen amides, and 7 other nitrogen compounds were used as a single nitrogen source. The assimilation of nitrogen by the yeast cell depended on the pattern of nitrogen assimilation. The NH_3 formation through enzymic hydrolysis of amides and urea hydrolysis was necessary before C. utilis absorbed these nitrogen sources to the cell for metabolic process. Peptone and urea had to pass to the transammination reaction before assimilated. The mechanism of assimilation of peptone and urea might be complicated than ammonium sulfate. Peptone is the soluble protein derivatives. It contains nitrogen in the form which readily available for the growth requirements. It has a high peptone and amino acid content and more complex nitrogenous constituents (Difco Manual, 1953; Morris, 1958). The structure of these peptide molecules cleary influence the assimilation and the growth of yeast (Suomalainen and Oura, 1971). Although it has been reviewed in literature that organic nitrogens such as peptone, urea, and other soluble protein derivatives are adequate and good source for yeast growth (Prescott and Dunn, 1959; Nickerson and Rose, 1956; Suomalainen and Oura, 1971), however, no yeast has yet been reported to have a specific requirement for an organic source of nitrogen (Nickerson and Rose, 1956).

An other disadvantage of peptone is that it might not provide enough sulfur for the good growth of the yeast. The sulfur in pineapple juice alone may not present in high concentration enough to cover the requirement by the yeast. Although the required concentration of sulfur for C. utilis has not been reported, Ammonium sulfate can supply the needed sulfur undoubtedly, but the amount of sulfur provided by peptone is suspicious as the structure of peptone is so complicated. Undoubtedly, urea cannot provide the required sulfur as can be seen from its structure. Beside the problem mentioned above, the disadvantage of urea comes from the limited concentration (0.1%) in the medium (Kurth, 1946). The concentration of urea might be optimum for particular strain of yeast. The exceedingly high concentration of urea would retard the growth of yeast (Kurth, 1946; Proscott and Dunn, 1959). Although urea has long been used to serve as a nitrogen source for production of C. utilis on many raw materials such as wood hydrolyzate (Harris et al., 1948; Kurth, 1946), sulfite liquor (Inskeep et al., 1951) and molasses (White, 1954; Nickerson and Rose, 1956; Peppler, 1970; Reed and Peppler, 1973). However, it was never used as a sole source of nitrogen but added in combination with ammonium sulfate and other nitrogen sources (Harris et al., 1948b, 1948c; Pyke, 1958). In this study, the concentration of urea used was not exceed the limit value according to the report of Kurth (1946) who stated that the concentration of urea should not excess of 0.05% for

beneficial results in respect to the growth of C. utilis. But it was reported by Prescott and Dunn (1959) that the concentration of urea should not exceed 0.092%. The present experiments were carried out to determine the growth of C. utilis on this two supplemental nitrogons (peptone and urea). The addition of yeast extract was found to improve the growth of yeast when peptone was used as nitrogen source (see Figure 6). Additional yeast extract perhaps eliminated the problem of peptone. Yeast extract may not only provide the amount of sulfur for yeast or even makes the sulfur absorbed to the cell of the yeast more easier. The result obtained in this study agrees with the work of Prescott and Dunn (1959) who reported that, in the presence of vitamin, yeast assimilated nitrogen source which otherwise partly assimilated. On the other hand, the growth of yeast in the medium 5 was poorer than in medium 4 (see Figure 7) although nitrogen source was provided by the mixture of ammonium sulfate and urea together with yeast extract in medium 5. Additional ammonium sulfate was intended to solve the problem of urea. It might provide sulfur which was lacking when urea was used as nitrogen source. Besides, the yeast could take up nitrogen from both ammonium sulfate and urea. Yeast extract was added to provide biotin which was required for the assimilation of urea (Suomalainen and Oura, 1971). Suomalainen and Oura (1971) also reported that, if the yeast was to grow in urea as well as in ammonium sulfate, the medium had to contain abundant amount of

biotin. So the nutrients that added in medium 5, were undoubtedly solved the problem of urea. The poor growth obtained in medium 5 could be explained as follows: (a) the concentration of ammonium sulfate and urea were not added in optimum proportion, (b) the competitive effect upon the two nitrogen sources, and (c) urea might exert the toxicity effect upon ammonium sulfate. The result is not agreed with Nickerson and Rose (1956) who reported that when yeast grown in a medium containing two nitrogen nutrients, there should have an increase in growth compared with a medium containing a single nitrogen source. The mixture of three compounds should still support higher growth than binary mixtures, whilo mixture containing four nitrogen sources should show still higher effect than ternary mixtures, and so on. The composition of medium 7 was said to fulfil the requirement of the yeast (Morris, 1958; Nickerson and Rose, 1956). In the present study, a mixture of three nutrient nitrogens, i.e., ammonium sulfate, peptone, and urea together with yeast extract were used. However, the yeast yield from medium 1 was better than medium 7 also with the protein content (see Table 3). There might be antagonistic effect in the medium 6 that sometimes resulted from growing yeast in the presence of a mixture of compounds eventhough the individual component of the mixture was beneficicial to the organism (Morris, 1958).

It appeared from the present work that, medium 1 gave better growth of C. utilis on pineapple juice than other media

studied on the values of the maximum yield and protein content obtained. The composition of medium 1 is suitable for commercial production of yeast as well. The main reason comes from the simple nutrient material in the medium. Ammonium sulfate was selected for nutrient nitrogen with many advantages as described earlier. There was evident in a well-balanced medium, that ammonium salts will support at least as much growth as any other single nitrogen sources. In general, ammonium nitrogen permits maximal growth of most yeasts. Most of food yeast of commerce has been produced with ammonium salt as the principal or even sole source of nitrogen (Frey et al., 1940; Nickerson and Rose, 1956; Morrison, 1958; Peppler, 1970).

5.2 Part II Cultivation of Candida utilis in Stirred-vessel Fermenter

The amount of air supplied to a fermenter and the manner of its distribution are well known to be critical factor in the efficiency of yeast production by any process. In order to obtain high aeration rate efficiency on a large scale, aeration must be accompanied with agitation of the medium (Wiley, 1954; Johnson and Olson, 1949). Agitation and aeration comprise a single effect because it is impossible to aerate all portion of a culture fluid without some degree of stirring (Finn, 1954). This step of the experiment was conducted in a stirred vessel

fermenter. Use of this apparatus was shown to give high rates of oxygen transfer, thus facilitating the better production of yeast (Harris et al., 1948c; Kurth, 1946; Frey et al., 1946). Since in higly stirred and aerated fermenter, microorganisms move regularly and fast enough to contact the substrate, it may be necessary to supply the substrate to all areas of the fermenter (Lefrancois and Revuz, 1973). Cell growth is stimulated by aeration with repress alcohol production. High degree of aeration efficiency and agitation permit very rapid utilization of sugar (Johnson and Olson, 1949; Reiser, 1954; Bunker, 1963). In this study, the speed of agitation was kept constant at the maximum value of 1400 rpm. Only aeration rate was varied to determine the influence of aeration on yeast growth, yield, protein content, sugar consumption and reduction of chemical oxygen demand as the function of cultivation time. The yeast was grown in medium 1 which was selected from the result in the part 1. Apart from a proper composition of the fermentation medium, the amount of air required for efficient yeast growth is known to be critical. Too much air can be accompanied by the increase of carbon dioxide respiration and heat formation, and low yeast yield. Too little air allows anaerobic fermentation condition to arise with the loss of yeast yields in favor of alcohol production (Wiley, 1954). Unfortunately, the efficiency of aeration is varied in different substrates and with differing types and sizes of equipment, hence it is rather

difficult to make generalized specification of air requirement for yeast. (Wiley, 1954; Finn, 1954; Solomoms, 1969). It has not yet been reported that yeast has an optimum aeration level. It must always be assumed that aeration limits the yield of yeast unless it can be shown that more effective aeration does not increase yields (Johnson and Olson, 1949). Change of aeration rate from 0.5 VVM to 1 VVM, better yield was obtained in this study (see Figure 10). But when the aeration rate was raised to 1.5 VVM, no improvement of the yield occured. So the optimum aeration rate obtained in the condition studied was 1 VVM judging from both growth curves and the yeast yields (see Table 4). The result agrees with Rhodes and Fletcher (1966), and Finn (1954) who reported that air rates should not exceed 70 feet per hour per gallon for fermenter with an open impellers or the maximum aeration rate should be 1 VVM. The yeast yield obtained from this study was in the range of 42-46% (see Table 3 and 4). The yeast yield obtained from the shake flask was 42.1% at the end of 64 hours, whereas the values of 49.1% was obtained when the yeast was grown in the same medium but in the stirred-vessel fermenter at the end of 8 hours. The important effect may be caused by aeration effect and cultivation time. Harris et al. (1948c) reported that on the production of C. utilis on wood hydrolyzates, the yield was about 42%. They also proposed that when yeasts grown for the production of cells, the dry cell yields obtained were approximately 45 percent of the substrate

utilized, and the cell concentration on a dry weight basis was 44 mg per 100 ml. Another agricultural waste product, i.e., protein waste water from a potato starch plant, was investigated by Reiser (1954) for its suitability to support the growth of C. utilis. The yeast yield was found to be about 45% whereas the same organism grown on molasses gave the yield about 56% (Burrows, 1970). In addition to the necessity of the balance composition of the medium, the yield of C. utilis was a function of the sugar present (Synder, 1970; Prescott and Dunn, 1959; Nolte et al., 1942). Cell yields of C. utilis grown on citrus press liquor varied from 70 to 26% depending on the initial concentration of sugar. The high cell yield of 70% was obtained with 0.6% initial sugar concentration, and the low yield of 25% occured with 3.8% sugar present initially (Synder, 1970). Nolte and his associates (1942) reported that when growed C. utilis on citrus-waste press juice, the yeast yield was varied with the concentration of total sugar content of the juice. The yield of C. utilis from the juice decreased from 44.3-48.0 to 37-47.7% when the total sugar content of the juice was increasel from 1 to 1.8%. It was also found that the use of a press juice containing not more than 1.0% total sugars seemed the most desirable because propagation was completed more quickly and yields were higher. Yield is always less than 100% because only part of the substrate consumed is recovered as cell mass. The remainder of the substrate is expended as by products of all metabolism, i.e.,

H_2O, CO_2, and other extracellular metabolized by-products. Not much change was obtained in the protein content of C. utilis grown in both shake flask and stirred-vessel fermenter (see Table 3 and 4). The average protein content obtained from yeast grown in medium 1 was about 55.38% on dry weight basis. Torulopsis utilis grown on citrus-waste press juice yielded a product with 55.28% protein (Nolte et al., 1942). This organism when grown on molasses yielded 55.9% protein (Ihl and Tagle, 1974) whereas the same organism when grown on the protein waste water from potato gave 55% protein (Reiser, 1954). In general, food yeast (C. utilis) grown on various raw materials such as sugar beet, sugar cane, molasses, sulfite liquor, potatoes, corn cobs, paraffin, etc. gave protein content of 45 to 57% on dry weight basis (Wuensche, 1967; Flannery, 1975). The protein content and the composition of amino acids depend on many factors, i.e., type of microorganisms, medium composition, and many physical conditions such as, temperature, pH, aeration and agitation (Tomas, 1973). Singh et al. (1948), and Vananuvat and Kinsella (1975) reported that aeration and agitation had negligible effects on the protein content of the food yeast. Yeast produced from sulfite waste liquor, mainly Torula type, can be used successfully in the food industry. Its protein, vitamins, amino acids, lecithin, macro and trace minerals [The inorganic substances essential for yeast may be divided into a macronutrient group (usually required at a concentration of

0.1-2.0 g/100 g dry weight of yeast) and a micronutrient group (required at a concentration of 0.5-10.0 mg/100 g dry weight of yeast). Thus, the macronutrients, including potassium, magnesium, phosphorus, sulfur are required in amounts approximately 200 times larger than the micronutrients (or trace elements) among which are iron, copper and zinc], and other useful components are in ample supply (Barta, 1971). Reyes and Cassas (1961) concluded that Candida utilis, propagated on waste of pineapple canning, contained significant amount of essential amino acids. The values obtained compared favorably with those found in food yeast grown on sulfite waste liquors. It was stated, however, that the protein quality of yeast grown on carbohydrates, regardless of sources (molasses, cellulose, sulfite waste liquor, etc.) would be superior than any other cheap proteins (Ghose, 1969). Generally, during cultivation of C. utilis at different aeration levels, the reduction of COD was obtained. In this study, about 78% COD reduction was obtained when C. utilis grown on pineapple juice supplemented with $(NH_4)_2SO_4$ 0.5% (w/v), and KH_2PO_4 0.5% (w/v) (see Figure 13). Yeast production was intended to be used in solving the waste disposal problem in many effuents of agricultural wastes, i.e., molasses, spent sulfite liquor, protein waste water such as corn steep liquor and potato wastes (Synder, 1970; Reed and Peppler, 1973; Kihlberg, 1972; Brock and Brock, 1973). The operating conditions and some limitings of the designed fermenter used in

this study did not permit the determination of optimum condition for yeast yield and COD reduction. Batch fermentation process generally has a very slow rate of growth at the start, know as induction period (Reiser, 1954). Continuous culture could give better control, shorter retention period, and lower operating costs (Sundhagul, 1972). Nevertheless, the preliminary results obtained in the present work serves to demonstrate the possibility of propagation Candida utilis on pineapple juice. With this technique dissolved materials such as sugar, phosphate, nitrogen, and many organic substances in the medium can be converted into yeast protein, which is attracting a growing interest as a contribution to the protein malnutrition. This process can lead to worthwhile utilization of organic nutrients in other waste effuents, thus providing a better means for lowering their pollution potential.